GLYCOSYLATION OF MOLECULES

Technical Field

The invention relates to methods of obtaining glycosylated molecules, particularly protein and lipid molecules.

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 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

The present ~~invention~~disclosure is based, at least in part, on: (a) the discovery that single gene deletion (Outer CHain elongation (OCH1) deletion) in *Yarrowia lypolitica* cells resulted in the substantially homogeneous production of glycosylated proteins having α-1,2-linked mannose residues on a $Man_5GlcNAc_2$ (structural formula IV; Fig. 1) backbone; (b) the discovery that overexpression of an engineered alpha-1,2-mannosidase targeted to the ER of *Yarrowia lipolytica* cells (both with AND without OCH1 deletion) resulted in the substantially homogenous production of glycosylated proteins carrying the $Man_5GlcNAc_2$ N-glycan structure (structural formula IV; Fig. 1); (c) the discovery that inactivating the Asparagine Linked Glycosylation 3 (ALG3) enzyme activity in *Yarrowia lipolytica* cells results in highly increased levels of glucosylated glycans; and (d) the discovery that overexpression of a wild-type form of a *Yarrowia lipolytica* gene (MNN4) in *Yarrowia lipolytica* results in hyperphosphorylation of α-1,2-linked mannose residues. Thus, the genetically engineered cells (e.g., *Yarrowia lipolytica*, *Arxula adeninivorans*, or other related species dimorphic yeast cells) can be used in methods to

produce target molecules having an altered N-glycosylation form as compared to the N-glycosylation form of the target molecules produced in non-genetically engineered cells of the same species. As administration of N-glycosylated target molecules (e.g., N-glycosylated proteins) to patients having a metabolic disorder (e.g., a lysosomal storage disorder) has been shown to ameliorate the symptoms of the disorder, the methods and cells described are useful for the preparation of N-glycosylated target molecules for the treatment of, *inter alia*, metabolic disorders such as lysosomal storage disorders.

The present ~~invention~~disclosure is also based, at least in part, on the discovery of the spliced form of the *Yarrowia lipolytica* and *Pichia pastoris* HAC1 gene. The protein encoded by the HAC1 gene, Hac1p, is a transcriptional activator that activates transcription of several target genes by binding to a DNA sequence motif termed the Unfolded Protein Response (UPR) element. Among the Hac1p target genes are those that encode chaperones, foldases, and proteins which are responsible for lipid-and inositol metabolism. As the spliced form Hac1p is a more potent transcriptional activator than the form encoded by the unspliced HAC1 mRNA, overexpression of the spliced form of Hac1p transcription factor can lead to an increased expression of native and heterologeous proteins as well as an increase in ER membrane. Thus, the spliced form of Hac1p can be used to increase the production of membrane and secreted proteins in a variety of eukaryotic cells (e.g., fungal cells (e.g., *Yarrowia lipolytica* or any other yeast cells described herein), plant cells, or animal cells (e.g., mammalian cells such as human cells) by simultaneous activation of the UPR and expression of target molecules.

The present ~~invention~~disclosure is further based on the discovery of a mutant form of the MNS1 mannosidase capable of converting $Man_8GlcNAc_2$ (structural formula I; Fig. 4) structures to $Man_5GlcNAc_2$ (structural formula IV; Fig. 4), $Man_6GlcNAc_2$ (structural formula V; Fig. 4) and $Man_7GlcNAc_2$ (structural formula VI; Fig. 4) when expressed in *Yarrowia lipolytica*. Thus, genetically engineered eukaryotic cells (e.g., fungal cells (e.g., *Yarrowia lipolytica* or any other yeast cells described herein), plant cells, or animal cells (e.g., mammalian cells such as human cells)) expressing mutant forms of mannosidase such as MNS1 can be used in methods to produce target molecules having an altered N-glycosylation form as compared to the N-glycosylation form of the target molecules produced in non-genetically engineered cells of the same species. Therefore, the cells and methods described are useful for the preparation of N-

glycosylated target molecules for the treatment of, *inter alia*, metabolic disorders such as lysosomal storage disorders (see below).

In one aspect, the present invention provides a method of producing an altered N-glycosylation form of a target protein or polypeptide, the method comprising:

providing a cell genetically engineered to comprise at least one modified N-glycosylation activity relative to a non-genetically engineered cell of the same species, the cell being a *Yarrowia lipolytica* or an *Arxula adeninivorans* cell or a cell from a related species of dimorphic yeast, the at least one modified N-glycosylation activity comprising:

expression of a phosphorylation-promoting protein or biologically active variant thereof that promotes mannosyl phosphorylation of the target protein or polypeptide, wherein the phosphorylation-promoting protein or biologically active variant thereof is MNN4 or PNO1; and

introducing into the cell a nucleic acid encoding a target protein or polypeptide, wherein the cell produces the target protein or polypeptide in an altered N-glycosylation form relative to a non-genetically engineered cell of the same species.

Also disclosed is~~disclosure features~~ a method of producing an altered N-glycosylation form of a target protein. The method includes the step of introducing into a cell a nucleic acid encoding a target protein, wherein the cell produces the target protein in an altered N-glycosylation form and wherein the cell is a *Yarrowia lipolytica* or an *Arxula adeninivorans* cell (or a related species dimorphic yeast cell) genetically engineered to contain at least one modified N-glycosylation activity. The method can also include the step of providing the *Yarrowia lipolytica* or an *Arxula adeninivorans* cell (or related species dimorphic yeast cell) genetically engineered to contain at least one modified N-glycosylation activity. The method can also include the step of isolating the altered N-glycosylation form of the target protein.

In some ~~embodiments~~examples, the target protein can be an endogenous protein or an exogenous protein. The target protein can be a mammalian protein such as a human protein. The target protein can be, for example, 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 fusion protein can be, for example, a fusion of a pathogen protein, a lysosomal protein, a growth factor, a cytokine, or a chemokine with an antibody or an antigen-binding fragment thereof. The target

protein can be, for example, one associated with a lysosomal storage disorder (LSD). The target protein can be, for example, glucocerebrosidase, galactocerebrosidase, alpha-L-iduronidase, beta-D-galactosidase, beta-glucosidase, beta-hexosaminidase, beta-D-mannosidase, alpha-L-fucosidase, arylsulfatase B, arylsulfatase A, alpha-N-acteylgalactosaminidase, aspartylglucosaminidase, iduronate-2-sulfatase, alpha-glucosaminide-N-acetyltransferase, beta-D-glucoronidase, hyaluronidase, alpha-L-mannosidase, alpha- neuraminidase, phosphotransferase, acid lipase, acid ceramidase, sphinogmyelinase, thioesterase, cathepsin K, or lipoprotein lipase.

In some ~~embodiments~~examples, the altered N-glycosylation form can contain one or more N-glycan structures such as, e.g., $Man_5GlcNAc_2$, $Man_8GlcNAc_2$, $Man_9GlcNAc_2$, $Man_3GlcNAc_2$, $Glc_1Man_5GlcNAc_2$, $Glc_2Man_5GlcNAc_2$. In some embodiments, the altered glycosylation can be, for example, $Man_5GlcNAc_2$, $Man_8GlcNAc_2$, $Man_9GlcNAc_2$, $Man_3GlcNAc_2$, $Glc_1Man_5GlcNAc_2$, $Glc_2Man_5GlcNAc_2$.

In some ~~embodiments~~examples, the altered N-glycosylation form of the target protein can be homogenous or substantially homogenous. For example, the fraction of altered target molecules that contain the altered glycosylation can be at least about 20%, at least about 30%, at least about 40%, at least about 45%, at least about 50%, at least about 55%, at least about 60%, at least about 65%, at least about 70%, at least about 75%, at least about 80%, at least about 85%, at least about 90%, or at least about 95% or more.

In some ~~embodiments~~examples, the cell can be genetically engineered to be deficient in at least one N-glycosylation activity. The N-glycosylation activity can be, for example, ALG3 activity, OCH1 activity, MNS1 activity, or MNN9 activity.

In some ~~embodiments~~examples, at least one modification can be: (a) deletion of a gene encoding a protein having the N-glycosylation activity; (b) expression of a mutant form of a protein having the N-glycosylation activity; (c) introduction or expression of an RNA molecule that interferes with the functional expression of a protein having the N-glycosylation activity; (d) expression of a protein having N-glycosylation activity (such as ALG6 or an alpha-mannosidase (e.g., an alpha-mannosidase targeted to the endoplasmic reticulum). The expressed protein can be a protein encoded by an exogenous nucleic acid in the cell. The expressed protein can be an alpha-mannosidase with a pH optimum below 7.5 (e.g., a pH optimum below 5.1). The protein having N-glycosylation activity can be an exogenous protein. The protein having N-glycosylation activity can be a mammalian protein (such as a human protein) or a lower eukaryotic (e.g., a fungus, a protozoan, or a trypanosome) protein. The lower eukaryote can be selected from the group consisting of *Typanosoma brucei*, *Trichoderma harzianum*, an *Aspergillus*, and any other lower eukaryote described herein.

In some ~~embodiments~~examples, the N-glycosylation activity can be a glucosyltransferase activity. In some embodiments, the protein having N-glycosylation activity is ALG6 or an alpha-mannosidase. The alpha-mannosidase can be targeted to the endoplasmic reticulum. For example, the protein having N-glycosylation activity can be a fusion protein comprising an alpha-mannosidase polypeptide and an HDEL endoplasmic reticulum retention peptide.

In some ~~embodiments~~examples, the protein having N-glycosylation activity can be a protein that is capable of removing glucose residues from $Man_5GlcNAc_2$. For example, the protein having N-glycosylation activity can be a protein having α-1,3-glucosidase activity such as, but not limited

to, a glucosidase II (e.g., one or both of the alpha and beta subunit of a glucosidase II) or a mutanase.

In some ~~embodiments~~examples, the cell can be genetically engineered to comprise at least two modified N-glycosylation activities such as any of the modified N-glycosylation activities described herein. The at least two modified N-glycosylation activities can comprise, e.g., a deficiency in an ALG3 activity and an elevated level of an ALG6 activity.

In some ~~embodiments~~examples, the cell can be genetically engineered to comprise at least three modified N-glycosylation activities such as any of the modified N-glycosylation activities described herein. The at least three modified N-glycosylation activities can comprise, e.g., a deficiency in an ALG3 activity; an elevated level of an ALG6 activity; and an elevated level of a a glucosidase II activity.

In some ~~embodiments~~examples, the cell is not genetically engineered to be deficient in an OCH1 activity.

In some ~~embodiments~~examples, modification can comprise expression of a protein or biologically active variant thereof capable of effecting mannosyl phosphorylation of the target protein. The protein or biologically active variant thereof capable of effecting mannosyl phosphorylation can be MNN4, PNO1, or MNN6. In some embodiments, at least about 30% of the mannosyl residues of a glycoprotein can be phosphorylated.

In some ~~embodiments~~examples, the method can further include additional processing of the glycoprotein. The additional processing can occur *in vitro* or *in vivo*. The additional processing can comprise addition of a heterologous moiety to the modified glycoprotein. The heterologous moiety can be a polymer or a carrier. The additional processing can comprise enzymatic or chemical treatment of the altered N-glycosylation form of the target protein. For example, the additional processing can comprise treatment of the altered N-glycosylation form of the target protein with a mannosidase, a mannanase, a phosphodiesterase, a glucosidase, or a glycosyltransferase. The additional processing can include treatment of the altered N-glycosylation form of the target protein with hydrofluoric acid. The additional processing can include phosphorylation of the altered N-glycosylation form of the target protein.

In another aspect, the present invention provides a method of producing an altered N-glycosylation form of a target protein, the method comprising:

contacting a target protein with a cell lysate prepared from a cell genetically engineered to comprise at least two modifications of N-glycosylation activity relative to a non-genetically engineered cell of the same species, the cell being a *Yarrowia lipolytica* or an *Arxula adeninivorans* cell or a cell from a related species of dimorphic yeast, the at least two modifications of N-glycosylation activity comprising: a deficiency in OCH1 activity; and

expression of a phosphorylation-promoting protein or biologically active variant thereof that promotes mannosyl phosphorylation of the target protein or polypeptide, wherein the phosphorylation-promoting protein or biologically active variant thereof is MNN4 or PNO1, and

wherein the contacting of the target protein with the cell lysate results in an altered N-glycosylation form of the target protein.

Also disclosed is~~disclosure provides~~ a method of producing an altered N-glycosylation form of a target protein. The method includes the steps of: providing a eukaryotic cell (e.g., a fungal cell, a plant cell, or an animal cell) genetically engineered to comprise at least

one modified N-glycosylation activity; and introducing into the cell a nucleic acid encoding a target protein, wherein the cell produces the target protein in an altered N-glycosylation form.

In another aspect, the present invention provides a method of producing an altered N-glycosylation form of a target protein, the method comprising contacting a target protein with one or more proteins having N-glycosylation activity, wherein the one or more proteins having N-glycosylation activity are obtained from a cell genetically engineered to comprise at least two modifications of N-glycosylation activity relative to a non-genetically engineered cell of the same species, the cell being a *Yarrowia lipolytica* or an *Arxula adeninivorans* cell or a cell from a related species of dimorphic yeast, the at least two modifications of N-glycosylation activity comprising:

a deficiency in OCH1 activity; and

expression of a phosphorylation-promoting protein or biologically active variant thereof that promotes mannosyl phosphorylation of the target protein or polypeptide, wherein the phosphorylation-promoting protein or biologically active variant thereof is MNN4 or PNO1, and

wherein contacting the target molecule with the one or more proteins having N-glycosylation activity results in an altered N-glycosylation form of the target protein.

Also disclosed is~~disclosure features~~ a method of producing an altered N-glycosylation form of a target protein. The method includes the step of contacting a target protein with a cell lysate prepared from a *Yarrowia lipolytica* or an *Arxula adeninivorans* cell genetically engineered to comprise at least one modified N-glycosylation activity, wherein the contacting of the target protein with the cell lysate results in an altered N-glycosylation form of the target protein.

Also disclosed is~~In yet another aspect, the disclosure features~~ a method of producing an altered N-glycosylation form of a target protein, which method includes the step of contacting a target protein with one or more proteins having N-glycosylation activity, wherein the one or more proteins having N-glycosylation activity are obtained from a *Yarrowia lipolytica* or an *Arxula adeninivorans* cell genetically engineered to comprise at least one modified N-glycosylation activity and wherein contacting the target molecule with the one or more proteins having N-glycosylation activity results in an altered N-glycosylation form of the target protein.

In another aspect, the present invention provides an isolated protein having altered N-glycosylation, wherein the protein is produced by the method as defined herein, and wherein at least 80% of the N-glycans of the protein are phosphorylated. Also disclosed is~~disclosure provides~~ an isolated protein having altered N-glycosylation, wherein the protein is produced by any of the methods described above.

In yet another aspect, the present invention provides a substantially pure culture of *Yarrowia lipolytica* or *Arxula adeninivorans* cells, a substantial number of which are genetically engineered to comprise at least two modifications of N-glycosylation activity comprising:

a deficiency in OCH1 activity; and expression of a phosphorylation-promoting protein or biologically active variant thereof that promotes mannosyl phosphorylation of the target protein or polypeptide, wherein the phosphorylation-promoting protein or biologically active variant thereof is MNN4 or PNO1.

Also disclosed is~~disclosure provides~~ an isolated *Yarrowia lipolytica* or *Arxula adeninivorans* cell (or other related species dimorphic yeast cell) genetically engineered to comprise at least one modified N-glycosylation activity. The N-glycosylation activity can be, for example, ALG3 activity, OCH1 activity, MNS1 activity, or MNN9 activity. The modification can be any of those described herein. For example, the modification can include: (a) deletion of a gene encoding a protein having the N-glycosylation activity, (b) expression of a mutant form of a protein having the N-glycosylation activity, (c) introduction or expression of an RNA molecule that interferes with the functional expression of a protein having the N-glycosylation activity, or (d) expression of a protein having N-glycosylation activity. The protein having N-glycosylation activity can be, for example, ALG6. The protein having N-glycosylation activity can be a mammalian protein such as a human protein. The modification can also include expression of a protein (e.g., MNN4 or PNO1) or biologically active variant thereof capable of promoting mannosyl phosphorylation of the modified glycoprotein.

In another aspect, the present invention provides the use of a protein as defined herein in the manufacture of a medicament for treating a disorder in a subject, the disorder is treatable by administration of a protein having altered N-glycosylation, and wherein the subject is one having, or suspected of having, a disease treatable by administration of a protein having altered N-glycosylation. Also disclosed is~~disclosure provides~~ a method of treating a disorder treatable by administration of a protein having altered N-glycosylation. The method includes the steps of administering to a subject a protein obtained by any of the methods described above, wherein the subject is one having, or suspected of having, a disease treatable by administration of a protein having altered N-glycosylation. The method can also include the steps of (a) providing a subject and/or (b) determining whether the subject has a disease treatable by administration of a protein having altered N-glycosylation. The subject can be mammal such as a human. The disorder can be, for example, a cancer, an immunological disorder (e.g., an inflammatory condition) or a metabolic disorder. The metabolic disorder can be any of those described herein, e.g., a lysosomal storage disorder (LSD) such as Gaucher disease, Tay-Sachs disease, Pompe disease, Niemann-Pick disease, or Fabry disease. The protein can be one associated with an LSD, e.g., the protein can be, for example, glucocerebrosidase, alpha-galactosidase. The protein can be, for example, alpha-L-iduronidase, beta-D-galactosidase, beta-glucosidase, beta-hexosaminidase, beta-D-mannosidase, alpha-L-fucosidase, arylsulfatase B, arylsulfatase A, alpha-N-acteylgalactosaminidase, aspartylglucosaminidase, iduronate-2-sulfatase, alpha-glucosaminide-N-acetyltransferase, beta-D-glucoronidase, hyaluronidase, alpha-L-mannosidase, alpha-neurominidase, phosphotransferase, acid lipase, acid ceramidase, sphinogmyelinase, thioesterase, cathepsin K, or lipoprotein lipase.

In another aspect, the present invention provides an isolated cell genetically engineered to comprise at least one modified N-glycosylation activity relative to a non-genetically engineered cell, the cell being a *Yarrowia lipolytica, or Arxula adeninivorans* cell or a cell from a related species of dimorphic yeast, the at least one modified N-glycosylation activity comprising expression of a phosphorylation-promoting protein or biologically active variant thereof that promotes mannosyl phosphorylation of the target protein or polypeptide, wherein the phosphorylation-promoting protein or biologically active variant thereof is MNN4 or PNO1.

Also disclosed is~~disclosure provides~~ a substantially pure culture of *Yarrowia lipolytica* or *Arxula adeninivorans* cells (or other related species dimorphic yeast cells), a substantial number of which being genetically engineered to comprise at least one modified N-glycosylation activity (such as any of the modifications described herein). The culture of cells can contain one or more subpopulations of cells, each subpopulation comprising a different modified glycosylation activity.

Also disclosed is~~In yet another aspect, the disclosure provides~~: (a) an isolated nucleotide sequence comprising SEQ ID NO:1 or SEQ ID NO:2; (b) an isolated nucleotide sequence comprising a sequence that is at least 80% identical to SEQ ID NO:1 or SEQ ID NO:2; or (c) a polypeptide encoded by the isolated nucleotide sequence of (a) or (b). In some ~~embodiments~~examples, the isolated nucleic acid sequence is SEQ ID NO:1 or SEQ ID NO:2.

Also disclosed is~~In another aspect, the disclosure features~~ an isolated nucleic acid containing: (a) a nucleotide sequence that hybridizes under highly stringent conditions to the complement of SEQ ID NO:1 or SEQ ID NO:2; or (b) the complement of the nucleotide sequence.

Also disclosed is~~In yet another aspect, the disclosure provides~~: (a) an isolated nucleotide sequence comprising (or consisting of) any of the nucleic acid sequences depicted herein; (b) an isolated nucleotide sequence comprising a sequence that is at least 80% identical to any of the nucleic acid sequences depicted herein; or (c) a polypeptide encoded by the isolated nucleotide sequence of (a) or (b). In some ~~embodiments~~examples, the isolated nucleic acid sequence is any of the nucleic acid sequences depicted herein.

Also disclosed is~~In another aspect, the disclosure features~~ an isolated nucleic acid containing: (a) a nucleotide sequence that hybridizes under highly stringent conditions to the complement of any of the nucleic acid sequences depicted herein; or (b) the complement of the nucleotide sequence.

Also disclosed is~~In yet another aspect, the disclosure provides~~: (a) a vector comprising any of the nucleic acid sequences described above or (b) a cultured cell containing the vector of (a). The vector can be an expression vector. The nucleic acid sequence in the vector can be operably linked to expression control sequence.

Also disclosed is~~In another aspect, the disclosure provides~~ a method for producing a protein. The method includes the step of culturing any of the cells described above under conditions permitting the expression of the polypeptide. The method can also include the step of ,after culturing the cell, isolating the polypeptide from the cell or the medium in which the cell was cultured. The cell can be, e.g., a cultured cell containing a vector comprising any of the nucleic acid sequences described above.

The target molecules (e.g., target proteins), proteins having N-glycosylation activity, and altered N-glycosylation molecules described herein (collectively referred to as "molecules of the invention") can, but need not, be isolated. The term "isolated" as applied to any of the molecules of the invention described herein refers to a molecule, or a fragment thereof, that has been separated or purified from components (e.g., proteins or other naturally-occurring biological or organic molecules) which naturally accompany it. It is understood that recombinant molecules (e.g., recombinant proteins) will always be "isolated." Typically, a molecule of the invention is

isolated when it constitutes at least 60%, by weight, of the total molecules of the same type in a preparation, e.g., 60% of the total molecules of the same type in a sample. For example, an

obtained from natural or recombinant sources (as opposed to direct, chemical synthesis), are highly specific to the target sequence, and are much slower to hybridize to the target than longer oligomers. Probes can be single or double stranded nucleic acid molecules.

In some ~~embodiments~~examples, a variant nucleic acid described herein can have a sequence comprising one or both strands with partial complementary (e.g., at least 50%, at least 60%, at least 70%, at least 80%, at least 90%, at least 95%, at least 96%, at least 97%, at least 98%, at least 99% complementary) to a region, portion, domain, or segment of the wild-type nucleic acid of interest (e.g., the HAC1 nucleic acid sequences as depicted in SEQ ID NO:1 or SEQ ID NO:2). In some ~~embodiments~~examples, a variant nucleic acid sequence of interest can have a sequence comprising one or both strands with full complementary (i.e., 100% complementary) to a region, portion, domain, or segment of the wild-type nucleic acid sequence. Sequence "complementarity" refers to the chemical affinity between specific nitrogenous bases as a result of their hydrogen bonding properties (i.e., the property of two nucleic acid chains having base sequences such that an antiparallel duplex can form where the adenines and uracils (or thymine, in the case of DNA or modified RNA) are apposed to each other, and the guanines and cytosines are apposed to each other). Fully complementary sequences, thus, would be two sequences that have complete one-to-one correspondence (i.e., adenine to uracil/thymidine and guanine to cytosine) of the base sequences when the nucleotide sequences form an antiparallel duplex.

Hybridization can also be used as a measure of homology between two nucleic acid sequences. A nucleic acid sequence described herein, or a fragment or variant thereof, can be used as a hybridization probe according to standard hybridization techniques. The hybridization of a certain probe of interest (e.g., a probe of a HAC1 nucleotide sequence, e.g., the HAC1 nucleotide sequences as depicted in SEQ ID NOS:1 or 2) to DNA or RNA from a test source (e.g., a eukaryotic cell) is an indication of the presence of DNA or RNA (e.g., a HAC1 nucleotide sequence) corresponding to the probe in the test source. Hybridization conditions are known to those skilled in the art and can be found in Current Protocols in Molecular Biology, John Wiley & Sons, N.Y., 6.3.1-6.3.6, 1991. Moderate hybridization conditions are defined as equivalent to hybridization in 2X sodium chloride/sodium citrate (SSC) at 30°C, followed by a wash in 1 X SSC, 0.1% SDS at 50°C. Highly stringent conditions are defined as equivalent to hybridization in 6X sodium chloride/sodium citrate (SSC) at 45°C, followed by a wash in 0.2 X SSC, 0.1% SDS at 65°C.