(12) INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

(19) World Intellectual Property Organization
International Bureau

(43) International Publication Date
18 September 2014 (18.09.2014)

WIPO | PCT

(10) International Publication Number
WO 2014/140927 A2

(51) International Patent Classification: Not classified

(21) International Application Number:
PCT/IB2014/001236

(22) International Filing Date:
13 February 2014 (13.02.2014)

(25) Filing Language: English

(26) Publication Language: English

(30) Priority Data:
61/764,502 13 February 2013 (13.02.2013) US

(71) Applicant: **LABORATOIRE FRANCAIS DU FRACTIONNEMENT ET DES BIOTECHNOLOGIES** [FR/FR]; 3, avenue des Tropiques, ZA Courtaboeuf, 91940 Les Ulis (FR).

(72) Inventors: **CHEN, Li-How**; 12 Wachusett Drive, Acton, MA 01720 (US). **MEADE, Harry M.**; 62 Grasmere Street, Newton, MA 02458 (US).

(74) Agents: **CHAJMOWICZ, Marion** et al.; Cabinet Becker & Associés, 25 rue Louis le Grand, F-75002 Paris (FR).

(81) Designated States *(unless otherwise indicated, for every kind of national protection available)*: AE, AG, AL, AM, AO, AT, AU, AZ, BA, BB, BG, BH, BN, BR, BW, BY, BZ, CA, CH, CL, CN, CO, CR, CU, CZ, DE, DK, DM, DO, DZ, EC, EE, EG, ES, FI, GB, GD, GE, GH, GM, GT, HN, HR, HU, ID, IL, IN, IR, IS, JP, KE, KG, KN, KP, KR, KZ, LA, LC, LK, LR, LS, LT, LU, LY, MA, MD, ME, MG, MK, MN, MW, MX, MY, MZ, NA, NG, NI, NO, NZ, OM, PA, PE, PG, PH, PL, PT, QA, RO, RS, RU, RW, SA, SC, SD, SE, SG, SK, SL, SM, ST, SV, SY, TH, TJ, TM, TN, TR, TT, TZ, UA, UG, US, UZ, VC, VN, ZA, ZM, ZW.

(84) Designated States *(unless otherwise indicated, for every kind of regional protection available)*: ARIPO (BW, GH, GM, KE, LR, LS, MW, MZ, NA, RW, SD, SL, SZ, TZ, UG, ZM, ZW), Eurasian (AM, AZ, BY, KG, KZ, RU, TJ, TM), European (AL, AT, BE, BG, CH, CY, CZ, DE, DK, EE, ES, FI, FR, GB, GR, HR, HU, IE, IS, IT, LT, LU, LV, MC, MK, MT, NL, NO, PL, PT, RO, RS, SE, SI, SK, SM, TR), OAPI (BF, BJ, CF, CG, CI, CM, GA, GN, GQ, GW, KM, ML, MR, NE, SN, TD, TG).

Published:

— *without international search report and to be republished upon receipt of that report (Rule 48.2(g))*

— *with sequence listing part of description (Rule 5.2(a))*

(54) Title: PROTEINS WITH MODIFIED GLYCOSYLATION AND METHODS OF PRODUCTION THEREOF

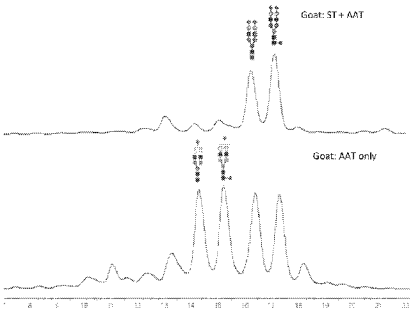


FIGURE 6

(57) Abstract: In one aspect, the disclosure provides proteins with modified glycosylation and methods of their production. In aspect, the disclosure provides transgenic animals and cells for the production of proteins with modified glycosylation. In some embodiment, the modified glycosylation is increased sialylation.

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PROTEINS WITH MODIFIED GLYCOSYLATION AND METHODS OF PRODUCTION THEREOF

RELATED APPLICATIONS

This application claims the benefit under 35 U.S.C. § 119(e) of U.S. Provisional Application Serial No. 61/764,502, entitled "Proteins with Modified Glycosylation and Methods of Production Thereof," filed on February 13, 2013, the entire disclosure of which is incorporated by reference herein in its entirety

FIELD OF THE INVENTION

The disclosure relates to the field of proteins and methods of protein production.

BACKGROUND OF THE INVENTION

Transgenic production of proteins in the mammary gland allows for the production of large amounts of proteins, including therapeutic proteins. However, the glycosylation pattern of proteins produced in the mammary gland sometimes differs from the glycosylation pattern of serum produced proteins. Methods to modify the glycosylation of mammary gland produced proteins are needed therefore.

SUMMARY OF THE INVENTION

In one aspect, the disclosure provides proteins with modified glycosylation and methods of their production. In aspect, the disclosure provides transgenic animals and cells for the production of proteins with modified glycosylation. In some embodiment, the modified glycosylation is increased sialylation.

In one aspect, the disclosure provides a method of producing protein with modified glycosylation, the method comprising providing a transgenic mammal that has been modified to have increased expression of sialyl transferase in the mammary gland, wherein the transgenic mammal expresses a protein in the mammary gland, and harvesting the protein from the mammary gland of the transgenic mammal.

In one aspect, the disclosure provides a method of producing protein with modified glycosylation, the method comprising providing a mammary epithelial cell that has been modified to have increased expression of sialyl transferase, wherein the mammary epithelial cell expresses a protein, and harvesting the protein from the mammary epithelial cells.

In some embodiments of the methods provided herein, the glycosylation is modified compared to a protein produced in a transgenic mammal or mammary epithelial cell that has not been modified to have increased expression of sialyl transferase. In some embodiments of the methods provided herein, the modified glycosylation is increased sialylation.

In one aspect, the disclosure provides a method of producing protein with increased sialylation, the method comprising providing a transgenic mammal that has been modified to have increased expression of sialyl transferase in the mammary gland, wherein the transgenic mammal expresses a protein in the mammary gland, and harvesting the protein with increased sialylation from the mammary gland of the transgenic mammal.

In some embodiments of the methods provided herein, the sialylation is increased compared to a protein produced in a transgenic mammal or mammary epithelial cell that has not been modified to have increased expression of sialyl transferase.

In some embodiments of the methods provided herein, the protein with modified glycosylation or protein with increased sialylation is a human protein. In some embodiments of the methods provided herein, the protein with modified glycosylation or protein with increased sialylation is an antibody. In some embodiments of the methods provided herein, the protein with modified glycosylation or protein with increased sialylation is antithrombin. In some embodiments of the methods provided herein, the protein with modified glycosylation or protein with increased sialylation is alpha-1 antitrypsin.

In one aspect, the disclosure provides a protein with modified glycosylation or protein with increased sialylation produced according to any of the methods provided herein.

In one aspect, the disclosure provides a composition comprising the protein with modified glycosylation or protein with increased sialylation produced according to any of the methods provided herein, the composition further comprising milk.

In one aspect, the disclosure provides a method of increasing the sialyl transferase activity in the mammary gland, the method comprising providing a transgenic mammal that has been modified to have increased expression of sialyl transferase in the mammary gland.

In one aspect, the disclosure provides a method of producing sialyl transferase, the method comprising providing a transgenic mammal that has been modified to have increased expression of sialyl transferase in the mammary gland, and harvesting the sialyl transferase from the mammary gland of the transgenic mammal. In some embodiments of the methods provided herein, the method further comprises using the sialyl transferase to sialylate a protein.

In one aspect, the disclosure provides a method of increasing the sialyl transferase activity in the mammary gland, the method comprising producing a transgenic mammal that has been modified to have increased expression of sialyl transferase in the mammary gland. In one aspect, the disclosure provides a method of producing sialyl transferase, the method comprising producing a transgenic mammal that has been modified to have increased expression of sialyl transferase in the mammary gland, and harvesting the sialyl transferase from the mammary gland of the transgenic mammal. In some embodiments of the methods provided herein, the method further comprises using the sialyl transferase to sialylate a protein. In some embodiments the sialyl transferase is placed in contact with the protein.

In one aspect, the disclosure provides a sialyl transferase produced according to any of the methods provided herein.

In one aspect, the disclosure provides a composition comprising the sialyl transferase produced according to any of the methods provided herein and further comprising milk.

In some embodiments of the methods, proteins, compositions, transgenic mammals or mammary gland epithelial cells provided herein, the transgenic mammal or mammary gland epithelial cell has been modified by introducing into the genome of the transgenic mammal or mammary gland epithelial cell a nucleic acid sequence encoding a sialyl transferase.

In some embodiments of the methods, proteins, compositions, transgenic mammals or mammary gland epithelial cells provided herein, the protein expressed in the transgenic mammal or mammary gland epithelial cell is encoded by a nucleic acid sequence introduced into the genome of the transgenic mammal or mammary gland epithelial cell.

In one aspect, the disclosure provides a transgenic mammal that has been modified to have increased expression of sialyl transferase in the mammary gland. In some embodiments of transgenic mammals provided herein, the transgenic mammal further expresses an exogenous protein in the mammary gland. In some embodiments of transgenic mammals provided herein, the exogenous protein is a human protein. In some embodiments of transgenic mammals provided herein, the exogenous protein is an antibody. In some embodiments of transgenic mammals provided herein, the exogenous protein is antithrombin. In some embodiments of transgenic mammals provided herein, the exogenous protein is alpha-1 antitrypsin.

In one aspect, the disclosure provides a mammary epithelial cell that has been modified to have increased expression of sialyl transferase. In some embodiments of the mammary epithelial cells provided herein, the mammary epithelial cell further expresses an exogenous protein. In some embodiments of the mammary epithelial cells provided herein,

the exogenous protein is a human protein. In some embodiments of the mammary epithelial cells provided herein, the exogenous protein is an antibody. In some embodiments of the mammary epithelial cells provided herein, the exogenous protein is antithrombin. In some embodiments of the mammary epithelial cells provided herein, the exogenous protein is alpha-1 antitrypsin.

In some embodiments of the methods, proteins, compositions, transgenic mammals or mammary gland epithelial cells provided herein, the transgenic mammal is an ungulate. In some embodiments, the ungulate is a goat.

In some embodiments of the methods, proteins, compositions, transgenic mammals or mammary gland epithelial cells provided herein, the expression of sialyl transferase is under the control of a milk promoter. In some embodiments, the milk promoter is a goat beta casein promoter.

In some embodiments of the methods, proteins, compositions, transgenic mammals or mammary gland epithelial cells provided herein, the sialyl transferase is beta-galactosamide alpha-2,6-sialyltranferase.

In some embodiments of the methods, proteins, compositions, transgenic mammals or mammary gland epithelial cells provided herein, the sialyl transferase is beta-galactosamide alpha-2,3-sialyltranferase.

Each of the limitations of the invention can encompass various embodiments of the invention. It is, therefore, anticipated that each of the limitations of the invention involving any one element or combinations of elements can be included in each aspect of the invention. This invention is not limited in its application to the details of construction and the arrangement of components set forth in the following description or illustrated in the Figures. The invention is capable of other embodiments and of being practiced or of being carried out in various ways. Also, the phraseology and terminology used herein is for the purpose of description and should not be regarded as limiting.

BRIEF DESCRIPTION OF THE DRAWINGS

The figures are illustrative only and are not required for enablement of the disclosure.

Figure 1 shows the sequence of ST6 beta-galactosamide alpha-2,6-sialyltranferase GENE ID: 100861310 ST6GAL1 [Capra hircus] (SEQ ID NO:1), top sequence labeled "Query", as compared to the human protein (SEQ ID NO:2), bottom sequence labeled "Subject".

Figure 2 provides a schematic representation of the ST6 beta-galactosamide alpha-2,6-sialyltransferase gene.

Figure 3 provides isoelectric focusing (IEF) gels showing proteins with increased levels of sialic acid. Figure 3A depicts two gels: The gel in the left panel shows a pI standard ladder (lane 1), alpha-antitrypsin (AAT; lane 2), and AAT that was treated with a mix of commercially available sialyl transferases (lane 3). The gel in the right panel depicts a pI standard ladder (lane 1), AAT before treatment with a sialyl transferase (lane 2), AAT after treatment with a mix of commercially available sialyl transferases (lane 3), or AAT after treatment with a single sialyl transferase purified from the milk of a transgenic goat (lanes 4 and 5). Figure 3B depicts a gel showing Alfalastin®, a sialylated version of AAT (lane 1), AAT treated with milk from sialyl transferase producing transgenic goats (lane 2 and lane 7), AAT treated with milk from a control animal (lanes 3 and 8), AAT treated with milk from a control animal and transgenically produced sialyl transferase (lane 4), AAT treated with buffer and transgenically produced sialyl transferase (lane 5), and AAT treated with buffer (lane 6).

Figure 4 provides a diagram showing the in vitro sialylation of alpha-1 antitrypsin with transgenically produced sialyl transferase.

Figure 5 shows the sialylation of lactoferrin in a transgenic goat that overexpresses sialyl transferase in the mammary gland (column 2) and a control animal (column 1).

Figure 6 shows the "in vivo" sialylation of AAT in the mammary gland of a goat producing both sialyl transferase and AAT (top graph) and AAT only (bottom graph).

Figure 7 shows the pharmacokinetic profiles of ST3 and ST6 sialylated transgenically produced-AAT compared to plasma-derived AAT (pdAAT).

DETAILED DESCRIPTION OF THE INVENTION

In one aspect, the disclosure provides proteins with modified glycosylation and methods of production thereof. In one aspect, the disclosure provides transgenic mammals and cells for the production of proteins with modified glycosylation.

In one aspect, the disclosure provides proteins with increased sialylation and methods of production thereof. In one aspect, the disclosure provides transgenic mammals and cells for the production of proteins with increased sialylation. In one aspect, the disclosure provides transgenic mammals and cells for the production of proteins with increased sialylation, wherein the transgenic mammal or cell has been modified to have increased expression of sialyl transferase.

Production of protein in the mammary gland of transgenic non-human mammals has the advantage of allowing for the production of large amounts of protein. A variety of proteins can be produced in the mammary gland and transgenic production in non-human mammals has been used to produce human therapeutic proteins, such as serum proteins. An example of a transgenically produced serum protein is Atryn®, a transgenically produced antithrombin, which has been approved for use in both the US and Europe (See *e.g.,* US 5,843,705, US 6,441,145, US 7,019,193 and US 7,928,064).

Proteins that are transgenically produced in the mammary gland have the same amino acid sequence as "natural" proteins, as long as the nucleic acid encoding the protein that is introduced into the transgenic mammal is the same as the "natural" protein. However, if the transgenic protein is a glycoprotein, there may be differences in the glycosylation pattern of proteins produced in the mammary gland of transgenic non-human mammals as compared to "natural" proteins. For instance, mammary gland produced proteins can have a lower level of sialylation compared to the same protein isolated from serum (See *e.g.,* Edmunds, T, et al., Blood 91(12), 1998, 4561-4571; Geun-Cheol Gil et al., Glycobiology 18(7), 2008, 526–539). Differences in glycosylation between recombinantly produced proteins and natural proteins are not unique to the production of recombinant (transgenic) protein in the mammary gland. Almost any production method of a recombinant protein *e.g.,* production in CHO cells, can result in a difference in glycosylation as compared to natural proteins, merely because of different availability and activity of glycosylation enzymes between cell types. A difference in glycosylation of the recombinantly produced protein may or may not be relevant. Recombinant glycoproteins, even therapeutic proteins, can still have the desired biological properties even if the glycosylation is different from the natural protein. In some instances, however, a difference in glycosylation may be preferred because the different glycosylation may provide the recombinantly produced proteins different functional properties as compared to the natural protein (*e.g.,* a slower clearing rate).

However, in some instances it is desirable that the glycosylation pattern of a recombinantly produced protein resemble, or be identical to, the glycosylation pattern of a natural protein (*e.g.,* a protein found in the blood stream).

A variety of methods are available to modify the glycosylation of recombinantly produced proteins. For instance, inhibitors of specific glycosylation enzymes can be added to the cells that are producing proteins resulting in a modified glycosylation of the produced proteins. In addition, glycosylated proteins can be modified post-production, for instance by

incubating the protein with one or more glycosylation enzymes and/or modifying the glycosylation through chemical reactions.

In one aspect, the disclosure provides methods of producing proteins with modified glycosylation. In one aspect, the disclosure provides transgenic mammals for the production of proteins with modified glycosylation. In some embodiments, the transgenic mammals allow for the production of proteins with modified glycosylation in the mammary gland.

In one aspect, the disclosure provides methods of producing proteins with modified glycosylation. In one aspect, the disclosure provides cells for the production of proteins with modified glycosylation. In some embodiments, the cells are mammary epithelial cells.

In one aspect, the disclosure provides proteins with modified glycosylation. In some embodiments the proteins are human proteins.

In one aspect, the disclosure provides a method of producing protein with modified glycosylation comprising providing or producing a transgenic mammal that has been/is modified to have increased expression of sialyl transferase in the mammary gland, wherein the transgenic mammal expresses a protein in the mammary gland, and harvesting the protein from the mammary gland of the transgenic mammal. In some embodiments, the glycosylation is modified compared to a protein produced in a transgenic mammal or mammary epithelial cell that has not been modified to have increased expression of sialyl transferase. In some embodiments, the modified glycosylation is increased sialylation.

In one aspect, the disclosure provides a method of producing protein with modified glycosylation comprising providing or producing a mammary epithelial cell that has been/is modified to have increased expression of sialyl transferase, wherein the mammary epithelial cell expresses a protein, and harvesting the protein from the mammary epithelial cells. In some embodiments, the glycosylation is modified compared to a protein produced in a transgenic mammal or mammary epithelial cell that has not been modified to have increased expression of sialyl transferase. In some embodiments, the modified glycosylation is increased sialylation.

In one aspect, the disclosure provides a method of producing protein with increased sialylation, the method comprising providing a transgenic mammal that has been modified to have increased expression of sialyl transferase in the mammary gland, wherein the transgenic mammal expresses a protein in the mammary gland, and harvesting the protein with increased sialylation from the mammary gland of the transgenic mammal. In some embodiments, the sialylation is increased compared to a protein produced in a transgenic mammal or mammary epithelial cell that has not been modified to have increased expression of sialyl transferase.

A protein with modified glycosylation, as used herein, refers to a protein having a glycosylation pattern resulting from production in the mammary gland of a transgenic mammal or mammary epithelial cells wherein the mammary gland or mammary epithelial cells has an increased expression of sialyl transferase(s). The glycosylation is modified compared to the same protein produced in the mammary gland of a transgenic mammal or mammary epithelial cell that does not have increased expression of sialyl transferase(s). In some embodiments, the modified glycosylation is increased sialylation (*i.e.,* an increased number of sialic acid groups on the oligomannose chains). In some embodiments, the sialylation of the protein is increased by 10%, by 20%, by 30%, by 40%, by 50%, by 60%, by 70%, by 80%, by 90%, or by 100% or more, compared to the same protein produced in the mammary gland of a transgenic mammal or mammary epithelial cell that does not have increased expression of sialyl transferases. Sialylation levels can be determined for instance by analyzing the glycosylation of the protein with HPLC/MS.

It should be appreciated that the level of sialylation is increased compared to the same protein produced in the mammary gland of a transgenic mammal or mammary epithelial cell that does not have increased expression of sialyl transferases. Proteins with increased sialylation, as defined herein, may still have lower levels of sialylation compared to "natural proteins" (*e.g.,* proteins produced in the liver). In some embodiments, the proteins with increased sialylation have a level of sialylation that is at least 10%, at least 20%, at least 30%, at least 40%, at least 50%, at least 60%, at least 70%, at least 80%, at least 90%, at least 100%, at least 200% or at least 300% or more, compared to the sialylation level in a "natural protein". As shown herein, the expression levels of sialyl transferases in the mammary gland and mammary epithelial cell is lower than the expression level in other organs and cells (*e.g.,* the liver). Accordingly, glycoproteins produced in mammary gland or mammary epithelial cell generally will have a lower level of sialylation than the same protein produced in other organs and cells (*e.g.,* the liver). This level may be lower even if the mammary gland or mammary epithelial cell has been modified to have increased expression of sialyl transferase according to the methods provided herein.

It should further be appreciated that production of a protein in the mammary gland of a transgenic mammal or mammary epithelial cell with increased expression of sialyl transferases may result in modified glycosylation that is not limited to increased sialylation. For instance, the increased availability of sialyl transferase may lead to oligosaccharide chains that are shorter because they are capped with sialic acid preventing further addition of monosaccharides. In addition, for instance, the increased availability of sialyl transferases

may lead to a different availability of substrates for glycosylation leading to the synthesis of oligosaccharide side chains with a different monosaccharide pattern.

It is envisioned that a variety of proteins with modified glycosylation (*e.g.,* increased sialylation) can be produced according to the methods provided herein. In some embodiments the protein is an exogenous protein (*i.e.,* a protein not naturally found in the genome of the transgenic mammal or mammary epithelial cell, such as a human protein in a transgenic goat). In some embodiments, the protein with modified glycosylation is a human protein. Proteins with modified glycosylation that can be produced according to the methods provided herein include antibodies and glycoproteins that are active in blood homeostasis, such as antithrombin and alpha-antitrypsin. In some embodiments, the protein "to be modified" is a transgenic protein that is expressed in the mammary gland of the transgenic mammal or mammary gland epithelial cell because it is encoded by a nucleic acid sequence introduced into the genome of the transgenic mammal or mammary gland epithelial cell. However, in some embodiments, the proteins with modified glycosylation that are produced according to the methods provided herein are proteins that are naturally expressed in the mammary gland or mammary epithelial cells, such as lactoferrin.

In one aspect, the disclosure provides a method of increasing the sialyl transferase activity in the mammary gland comprising providing a transgenic mammal that has been modified to have increased expression of sialyl transferase in the mammary gland. In one aspect, the disclosure provides a method of increasing the sialyl transferase activity in mammary epithelial cells comprising providing or producing a mammary epithelial cell that has been/is modified to have increased expression of sialyl transferase. Increased expression, as used herein, refers to an increase in expression compared to an unmodified ("natural") mammary gland or unmodified mammary epithelial cell. It should be appreciated, and also shown herein, that an unmodified mammary gland or unmodified mammary epithelial cell has some residual expression of sialyl transferase. Thus, in some embodiments, the increased expression of sialyl transferase in the mammary gland or mammary epithelial cells is 2x higher or more, 3x higher or more, 4x higher or more, 5x higher or more, 10x higher or more, 20x higher or more, 50x higher or more, 100x higher or more, or 1000x higher or more than the natural expression of sialyl transferase in the mammary gland or mammary epithelial cells.

The mammary gland or mammary epithelial cells can be modified in a variety of ways to increase expression of sialyl transferase. In some embodiments, the expression is increased by upregulating the expression of one or more sialyl transferases that are naturally

present in the genome. In some embodiments, the expression is increased by introducing a new mammary specific promoter into the genome upstream of the one or more sialyl transferases that are naturally present in the genome. In some embodiments, the transgenic mammal or mammary gland epithelial cell has been modified by introducing into the genome of the transgenic mammal or mammary gland epithelial cell a nucleic acid sequence encoding a sialyl transferase. It should be appreciated that the transgenic mammals and mammary epithelial cells that have been modified to express sialyl transferase may also express a protein to be modified (*e.g.,* an exogenous protein).

Sialyl transferases are members of the glycosyltransferases group of enzymes. Sialyl transferase is an enzyme that can transfer sialic acid onto an oligosaccharide, including oligosaccharides found on glycosylated proteins. In general sialyl transferases introduced sialic acids on the terminal positions of glycoconjugate sugar chains. Sialyl transferases are categorized according to their specific activity, and are generally classified in three groups: alpha-2,6-sialytransferases, alpha-2,3-sialytransferases and alpha-2,8-sialytransferases, which can introduce sialic acid groups through an alpha-2,6-, alpha-2,3-, and alpha-2,8- linkage, respectively (See *e.g.,* Harduin-Lepers et al., The human sialyltransferase family, Biochimie 83: 727-737, 2001).

The alpha-2,6-sialytransferases are further classified in sialyl transferases that attach a sialic acid to galactose ("ST6Gal I", "beta-galactosamide alpha-2,6-sialyltransferase" or ST6 beta-galactosamide alpha-2,6-sialyltransferase 1"), and sialyl transferases that attach a sialic acid to GalNAc ("ST6GalNAc I-VI"). The beta-galactosamide alpha-2,6-sialyltransferase mediates the transfer of a sialic acid residue to a terminal Gal residue on a disaccharide found as a free disaccharide or found as a terminal unit on a N-linked or O-linked oligosaccharide. Thus, in some embodiments, the disclosure provides methods and compositions that allow for N-linked sialylation. In some embodiments, the disclosure provides methods and compositions that allow for O-linked sialylation.

The GalNAc alpha-2,6-sialyltransferases can attach sialic acid to non-terminal GalNAc residues.

The alpha-2,3 sialyltransferases mediate the transfer of sialic acid though a 2,3 linkage to a terminal Gal residue on glycolipids and glycoproteins.

The alpha-2,8 sialyltransferases generally are involved in the generation of poly-sialic acid chains.

In some embodiments, the sialyl transferase is beta-galactosamide alpha-2,6-sialyltransferase ("ST6 Gal"), alpha-N-acetylgalactosaminide alpha-2,6-sialyltransferase,

beta-galactoside alpha-2,3-sialyltransferase, N-acetyllactosaminide alpha-2,3-sialyltransferase, alpha-N-acetyl-neuraminide alpha-2,8-sialyltransferase, or lactosylceramide alpha-2,3-sialyltransferase. In some embodiments the sialyl transferase is beta-galactosamide alpha-2,6-sialyltransferase ("ST6 Gal"). In some embodiments, the sialyl transferase is beta-galactoside alpha-2,3-sialyltransferase. Sequences of human sialyl transferases are provided, for instance, in Harduin-Lepers et al., (The human sialyltransferase family, Biochimie 83: 727-737, 2001)

In general the species of sialyl transferase used according to the methods of the invention will be the same as species of transgenic mammal or the mammary epithelial cell. For instance, goat sialyl transferase is introduced in a transgenic goat or goat mammary epithelial cells. However, it should be appreciated that the sialyl transferase used may also be from a different species as the transgenic mammal or the mammary epithelial. In some embodiments, the sialyl transferase introduced into the transgenic mammal or the mammary epithelial cell is from the same species as the protein to be modified (*e.g.,* a human sialyl transferase is used to generate a human antibody with increased sialylation). In some embodiments, the transgenic mammal or the mammary epithelial cell transgenically expresses a sialyl transferase and an exogenous protein, from the same species (*e.g.,* a goat is transgenic for both human sialyl transferase and a human therapeutic antibody).

In one aspect, the disclosure provides methods and compositions to treat inflammation. It has been shown that the anti-inflammatory activity of intravenously administered Ig results from a subset of the IgG molecules that have terminal alpha-2,6-sialic acid linkages on their Fc-linked glycans. The anti-inflammatory activity of the population of the intravenously administered Ig was increased by introducing a terminal alpha-2,6-sialic acid linkage on all Fc-linked glycans (See *e.g.,* Anthony et al., Identification of a receptor required for the anti-inflammatory activity of IVIG, PNAS 105: 19571-19578, 2008). The methods provided herein allow for the production in the mammary gland of transgenic animals and mammary epithelial cells of exogenous proteins, such as therapeutic antibodies, with increased levels of alpha-2,6-sialylation. Thus, the methods provided herein allow for the production in the mammary gland of transgenic animals and mammary epithelial cells of exogenous proteins, such as therapeutic antibodies, with increased anti-inflammatory properties.

It should be appreciated that the methods provided herein to increase the anti-inflammatory properties of therapeutic antibodies can be applied to any therapeutic antibody. Thus, in some embodiments, the disclosure provides therapeutic antibodies with increased

anti-inflammatory activity. It should further be appreciated that the methods provided for the generation of antibodies with increased anti-inflammatory activity may be applied to anti-inflammatory antibodies, thereby providing anti-inflammatory antibodies with synergistic mode of actions (through binding to an anti-inflammatory target and through the alpha-2,6-sialic acid linkages on their Fc-linked glycans). Therapeutic antibodies for anti-inflammatory treatment include anti-TNF alpha antibodies such as infliximab / Remicade (Centocor) a mouse-human chimeric monoclonal anti TNF-antibody, adalimumab / Humira (Abbott) a fully human anti-TNF antibody, and golimumab / Simponi (Centocor) a fully human anti-TNF antibody, therapeutic TNF-alpha binders that are antibody-based including Etanercept / Enbrel (Amgen) a fusion protein of the extracellular domain of TNF-receptor fused to the Fc region of Ig1, and certolizumab pegol / Cimzia (UCB) a Pegylated Fab' fragment of humanized monoclonal anti-TNF antibody. Therapeutic antibodies for anti-inflammatory treatment also include non-TNF-alpha anti-cytokine antibodies such as anti-IL5 (*e.g.,* mepolizumab), anti-IL2 (*e.g.,* daclizumab), anti-IL4 and anti-IL13 antibodies. Therapeutic antibodies for anti-inflammatory treatment also include anti-IgE antibodies (*e.g.,* omalizumab), anti-CD62 antibodies, and others.

Thus, in one aspect, the disclosure provides transgenic animals (and mammary epithelial cells) that are transgenic for the production in the mammary gland of an exogenous therapeutic antibody and that are transgenic for the production of sialyl transferase. The therapeutic antibodies produced by such animals and cells have increased levels of terminal alpha-2,6-sialic acid linkages on their Fc-linked glycans, and thereby have increased anti-inflammatory activity (as compared to therapeutic antibodies produced in transgenic animals and mammary cells that are not transgenic for the expression of sialyl transferase). In some embodiments, the transgenic animals (and mammary epithelial cells) are transgenic for the production in the mammary gland of an exogenous anti-inflammatory antibody and are transgenic for the production of sialyl transferase. In some embodiments, the anti-inflammatory antibody is an anti-TNF-alpha antibody. In some embodiments, the anti-inflammatory antibody is adalimumab.

In one aspect, the disclosure provides methods of treating inflammation in a subject comprising administering to a subject the antibodies with increased anti-inflammatory properties provided herein.

In one aspect, the disclosure provides a method of producing sialyl transferase comprising providing or producing a transgenic mammal that has been/is modified to have increased expression of sialyl transferase in the mammary gland, and harvesting the sialyl

transferase from the mammary gland of the transgenic mammal. In some embodiments, the transgenic mammal or mammary gland epithelial cell has been modified by introducing into the genome of the transgenic mammal or mammary gland epithelial cell a nucleic acid sequence encoding a sialyl transferase.

In some embodiments, the sialyl transferase produced according to the methods provided herein is used to sialylate a protein.

In one aspect, the disclosure provides a sialyl transferase produced by providing a transgenic mammal that has been modified to have increased expression of sialyl transferase in the mammary gland, and harvesting the sialyl transferase from the mammary gland of the transgenic mammal. In some embodiments, the transgenic mammal has been modified by introducing into the genome of the transgenic mammal a nucleic acid sequence encoding a sialyl transferase.

In some embodiments, the sialyl transferase is in a composition that further comprises milk.

In one aspect, the disclosure provides a protein with modified glycosylation produced by providing or producing a transgenic mammal that has been/is modified to have increased expression of sialyl transferase in the mammary gland, wherein the transgenic mammal expresses the protein in the mammary gland and harvesting the protein with modified glycosylation from the mammary gland of the transgenic mammal. In some embodiments, the transgenic mammal has been modified by introducing into the genome of the transgenic mammal cell a nucleic acid sequence encoding a sialyl transferase.

In some embodiments, the protein with modified glycosylation is in a composition that further comprises milk. In some embodiments, the protein with modified glycosylation is a human protein. In some embodiments, the protein with modified glycosylation is an antibody, antithrombin or alpha antitrypsin.

Transgenic mammals

In one aspect, the disclosure provides a transgenic mammal that has been modified to have increased expression of sialyl transferase in the mammary gland. In some embodiments, the transgenic mammal is an ungulate. In some embodiments, the ungulate is a goat.

In one aspect, the disclosure provides a mammary epithelial cell that has been modified to have increased expression of sialyl transferase.

In some embodiments, the transgenic mammal or mammary gland epithelial cell has been modified by introducing into the genome of the transgenic mammal or mammary gland epithelial cell a nucleic acid sequence encoding a sialyl transferase. In some embodiments, the expression of sialyl transferase is under the control of a milk promoter. In some embodiments, the milk promoter is a goat beta casein promoter.

In one aspect, the disclosure provides methods of producing proteins with modified glycosylation, wherein the protein to be modified is expressed in the mammary gland or mammary epithelial cells. In some embodiments, the protein expressed in the transgenic mammal or mammary gland epithelial cell is encoded by a nucleic acid sequence introduced into the genome of the transgenic mammal or mammary gland epithelial cell. In some embodiments, the expression of sialyl transferase is under the control of a milk promoter. In some embodiments, the milk promoter is a goat beta casein promoter.

In one aspect, the disclosure provides a transgenic non-human mammal that has increased expression of sialyl transferase in the mammary gland. In some embodiments, the transgenic non-human mammal that has increased expression of sialyl transferase in the mammary gland also expresses a transgenic protein in the mammary gland (*e.g.,* an exogenous protein).

In one aspect, the disclosure provides mammary gland epithelial cells that have increased expression of sialyl transferase. In some embodiments, the mammary gland epithelial cells that have increased expression of sialyl transferase also express a recombinant protein (*e.g.,* an exogenous protein).

In one aspect, the disclosure provides a method of production of sialyl transferase comprising expressing in the milk of a transgenic non-human mammal having a sialyl transferase encoded by a nucleic acid construct. In one aspect, the disclosure provides a method of the production of a protein with modified glycosylation comprising expressing in the milk of a transgenic non-human mammal that has increased expression of sialyl transferase in the mammary gland and a transgenic protein (*e.g.,* an exogenous protein) encoded by a nucleic acid construct.

In some embodiments, the method of producing the sialyl transferase or protein with modified glycosylation comprises:

(a) transfecting non-human mammalian cells with a transgene DNA construct encoding sialyl transferase and/or the protein to be modified;

(b) selecting cells in which said transgene DNA construct(s) has/have been inserted into the genome of the cells; and

(c) performing a first nuclear transfer procedure to generate a non-human transgenic mammal heterozygous for sialyl transferase and/or protein to be modified and that can express the sialyl transferase and or protein with modified glycosylation in its milk, and, optionally

(d) repeating one or more of the steps to create a transgenic mammal that can express both the sialyl transferase and protein to be modified in the mammary gland. In one aspect, the disclosure provides a method of:

(a) providing a non-human transgenic mammal engineered to express sialyl transferase and protein to be modified (*e.g.,* an exogenous protein).

(b) expressing sialyl transferase and protein to be modified in the milk of the non-human transgenic mammal; and

(c) isolating the protein with modified glycosylation produced in the milk.

Transgenic mammals can also be generated according to methods known in the art (See *e.g.*, U.S. Patent No. 5,945,577). Mammals suitable for transgenic expression, include, but are not limited to goat, sheep, bison, camel, cow, pig, rabbit, buffalo, horse, rat, mouse or llama. Suitable mammals also include bovine, caprine, ovine and porcine, which relate to various species of cows, goats, sheep and pigs (or swine), respectively. Suitable mammals also include ungulates. As used herein, "ungulate" is of or relating to a hoofed typically herbivorous quadruped mammal, including, without limitation, sheep, swine, goats, cattle and horses. In one embodiment, the mammals are generated by co-transfecting primary cells with separate constructs. These cells are then used for nuclear transfer. Alternatively, if micro-injection is used to generate the transgenic mammals, the constructs may be injected.

Mammals that are transgenic for one protein (*e.g.,* sialyl transferase) may be crossed with mammals that are transgenic for the protein to be modified, thereby generating mammals that are transgenic both for sialyl transferase and for the protein to be modified.

Cloning will result in a multiplicity of transgenic mammals – each capable of producing a sialyl transferase or other gene construct of interest. The production methods include the use of the cloned mammals and the offspring of those mammals. In some embodiments, the cloned mammals are caprines, bovines or mice. Cloning also encompasses the nuclear transfer of fetuses, nuclear transfer, tissue and organ transplantation and the creation of chimeric offspring.

One step of the cloning process comprises transferring the genome of a cell that contains the transgene encoding the sialyl transferase and/or protein to be modified into an enucleated oocyte. As used herein, "transgene" refers to any piece of a nucleic acid molecule

that is inserted by artifice into a cell, or an ancestor thereof, and becomes part of the genome of a mammal which develops from that cell. Such a transgene may include a gene which is partly or entirely exogenous (*i.e.,* foreign) to the transgenic mammal, or may represent a gene having identity to an endogenous gene of the mammal.

Suitable mammalian sources for oocytes include goats, sheep, cows, pigs, rabbits, guinea pigs, mice, hamsters, rats, non-human primates, etc. Preferably, oocytes are obtained from ungulates, and most preferably goats or cattle. Methods for isolation of oocytes are well known in the art. Essentially, the process comprises isolating oocytes from the ovaries or reproductive tract of a mammal, *e.g.,* a goat. A readily available source of ungulate oocytes is from hormonally-induced female mammals. For the successful use of techniques such as genetic engineering, nuclear transfer and cloning, oocytes may preferably be matured *in vivo* before these cells may be used as recipient cells for nuclear transfer, and before they were fertilized by the sperm cell to develop into an embryo. Metaphase II stage oocytes, which have been matured *in vivo,* have been successfully used in nuclear transfer techniques. Essentially, mature metaphase II oocytes are collected surgically from either non-super ovulated or super ovulated mammals several hours past the onset of estrus or past the injection of human chorionic gonadotropin (hCG) or similar hormone.

One of the tools used to predict the quantity and quality of the recombinant protein expressed in the mammary gland is through the induction of lactation (Ebert KM, 1994). Induced lactation allows for the expression and analysis of protein from the early stage of transgenic production rather than from the first natural lactation resulting from pregnancy, which is at least a year later. Induction of lactation can be done either hormonally or manually.

In some embodiments, the compositions of sialyl transferase and/or protein with modified glycosylation produced according to the methods provided herein further comprise milk.

In some embodiments the methods provided herein include a step of harvesting (*e.g.,* isolating) the sialyl transferase and/or protein with modified glycosylation from the milk of a transgenic mammal (See *e.g.*, Pollock et al., Journal of Immunological Methods, Volume 231, Issues 1-2, 10 December 1999, Pages 147-157).

Constructs for the generation of transgenic mammals

In some embodiments, to produce primary cell lines containing a construct (*e.g.,* encoding sialyl transferase and/or protein to be modified) for use in producing transgenic mammals (*e.g.,* goats) by nuclear transfer, the constructs can be transfected into primary (goat) skin epithelial cells, which are clonally expanded and fully characterized to assess transgene copy number, transgene structural integrity and chromosomal integration site. As used herein, "nuclear transfer" refers to a method of cloning wherein the nucleus from a donor cell is transplanted into an enucleated oocyte.

Coding sequences for proteins of interest (*e.g.,* sialyl transferase) can be obtained by screening libraries of genomic material or reverse-transcribed messenger RNA derived from the mammal of choice (such as an ungulate, rodent, etc.), obtained from sequence databases such as NCBI, Genbank, or by obtaining the sequences of sialyl transferase and/or protein to be modified, etc. The sequences can be cloned into an appropriate plasmid vector and amplified in a suitable host organism, like *E. coli.* After amplification of the vector, the DNA construct can be excised, purified from the remains of the vector and introduced into expression vectors that can be used to produce transgenic mammals. The transgenic mammals will have the desired transgenic protein integrated into their genome.

After amplification of the vector, the DNA construct can be excised with the appropriate 5' and 3' control sequences, purified away from the remains of the vector and used to produce transgenic mammals that have integrated into their genome the desired non-glycosylated related transgenic protein. Conversely, with some vectors, such as yeast artificial chromosomes (YACs), it is not necessary to remove the assembled construct from the vector; in such cases the amplified vector may be used directly to make transgenic mammals. The coding sequence can be operatively linked to a control sequence, which enables the coding sequence to be expressed in the milk of a transgenic non-human mammal.

A DNA sequence which is suitable for directing production of sialyl transferase and/or protein to be modified to the milk of transgenic mammals can carry a 5'-promoter region derived from a naturally-derived milk protein. This promoter is consequently under the control of hormonal and tissue-specific factors and is most active in lactating mammary tissue. In some embodiments, the promoter is a caprine beta casein promoter. The promoter can be operably linked to a DNA sequence directing the production of a protein leader sequence, which directs the secretion of the transgenic protein across the mammary epithelium into the milk. In some embodiments, a 3'-sequence, which can be derived from a naturally secreted milk protein, can be added to improve stability of mRNA.

As used herein, a "leader sequence" or "signal sequence" is a nucleic acid sequence that encodes a protein secretory signal, and, when operably linked to a downstream nucleic acid molecule encoding a transgenic protein directs secretion. The leader sequence may be the native human leader sequence, an artificially-derived leader, or may obtained from the same gene as the promoter used to direct transcription of the transgene coding sequence, or from another protein that is normally secreted from a cell, such as a mammalian mammary epithelial cell.

In some embodiments, the promoters are milk-specific promoters. As used herein, a "milk-specific promoter" is a promoter that naturally directs expression of a gene in a cell that secretes a protein into milk (*e.g.,* a mammary epithelial cell) and includes, for example, the casein promoters, *e.g.*, α-casein promoter (*e.g.*, alpha S-1 casein promoter and alpha S2-casein promoter), β-casein promoter (*e.g.*, the goat beta casein gene promoter (DiTullio, BIOTECHNOLOGY 10:74-77, 1992), γ-casein promoter, κ-casein promoter, whey acidic protein (WAP) promoter (Gordon et al., BIOTECHNOLOGY 5: 1183-1187, 1987), β-lactoglobulin promoter (Clark et al., BIOTECHNOLOGY 7: 487-492, 1989) and α-lactalbumin promoter (Soulier et al., FEBS LETTS. 297:13, 1992). Also included in this definition are promoters that are specifically activated in mammary tissue, such as, for example, the long terminal repeat (LTR) promoter of the mouse mammary tumor virus (MMTV).

As used herein, a coding sequence and regulatory sequences are said to be "operably joined" when they are covalently linked in such a way as to place the expression or transcription of the coding sequence under the influence or control of the regulatory sequences. In order for the coding sequences to be translated into a functional protein the coding sequences are operably joined to regulatory sequences. Two DNA sequences are said to be operably joined if induction of a promoter in the 5' regulatory sequences results in the transcription of the coding sequence and if the nature of the linkage between the two DNA sequences does not (1) result in the introduction of a frame-shift mutation, (2) interfere with the ability of the promoter region to direct the transcription of the coding sequences, or (3) interfere with the ability of the corresponding RNA transcript to be translated into a protein. Thus, a promoter region is operably joined to a coding sequence if the promoter region were capable of effecting transcription of that DNA sequence such that the resulting transcript might be translated into the desired protein or polypeptide.

As used herein, a "vector" may be any of a number of nucleic acids into which a desired sequence may be inserted by restriction and ligation for transport between different

genetic environments or for expression in a host cell. Vectors are typically composed of DNA although RNA vectors are also available. Vectors include, but are not limited to, plasmids and phagemids. A cloning vector is one which is able to replicate in a host cell, and which is further characterized by one or more endonuclease restriction sites at which the vector may be cut in a determinable fashion and into which a desired DNA sequence may be ligated such that the new recombinant vector retains its ability to replicate in the host cell. In the case of plasmids, replication of the desired sequence may occur many times as the plasmid increases in copy number within the host bacterium, or just a single time per host as the host reproduces by mitosis. In the case of phage, replication may occur actively during a lytic phase or passively during a lysogenic phase. An expression vector is one into which a desired DNA sequence may be inserted by restriction and ligation such that it is operably joined to regulatory sequences and may be expressed as an RNA transcript. Vectors may further contain one or more marker sequences suitable for use in the identification of cells, which have or have not been transformed or transfected with the vector. Markers include, for example, genes encoding proteins which increase or decrease either resistance or sensitivity to antibiotics or other compounds, genes which encode enzymes whose activities are detectable by standard assays known in the art (*e.g.,* ß-galactosidase or alkaline phosphatase), and genes which visibly affect the phenotype of transformed or transfected cells, hosts, colonies or plaques. Preferred vectors are those capable of autonomous replication and expression of the structural gene products present in the DNA segments to which they are operably joined.

The present invention is further illustrated by the following Examples, which in no way should be construed as further limiting. The entire contents of all of the references (including literature references, issued patents, published patent applications, and co-pending patent applications) cited throughout this application are hereby expressly incorporated by reference, in particular for the teaching that is referenced hereinabove. However, the citation of any reference is not intended to be an admission that the reference is prior art.

Examples

Example 1

Determining the level of sialyl transferase in the mammary gland

A reason for the incomplete sialylation of proteins produced in the mammary gland could be the limitation in the level of sialyl transferase in the mammary gland as compared to the level of sialyl transferase found in the liver, where serum proteins are normally secreted. To evaluate this hypothesis, the gene for 2,6 sialyl transferase was isolated from a cDNA library from goat liver. This cDNA was used as a probe in a Northern blot to determine the level of expression of the gene in the mammary gland vs. the liver. By Northern analysis, the level found of sialyl transferase expression in the mammary gland is about one percent of that found in the liver, implying a limitation in the sialylation capacity in the mammary gland.

Cloning of ST6 Gal1 from goat liver

A cDNA library was constructed using RNA isolated from goat liver. The known sequence of human ST6 was used to design PCR primers that could amplify the goat sequence. Following RT-PCR reactions, a full-length cDNA encoding 2,6 sialyl transferase, (ST6 Gal1), was isolated. The protein sequence of goat ST6 Gal1 is comparable to that of the human protein (See Figure 1: GENE ID: 100861310 ST6GAL1, ST6 beta-galactosamide alpha-2,6-sialyltranferase-1 [Capra hircus]; Figure 1 depicts both the goat (top: "Query"; SEQ ID NO:1) and human sequence of ST6 Gal1 (bottom: "Subject"; SEQ ID NO:2)

The gene encoding the 2,6 sialyl transferase was ligated into the goat beta casein expression vector to yield the plasmid BC2541 ST6Gal1 (See Figure 2). This vector includes the goat beta-casein promoter to direct expression of linked genes into the mammary gland.

Generation of transgenic goats

The BC2541 ST6Gal1 transgene was used to generate transgenic goats that express the ST6Gal1 in their mammary gland. The resulting goats carry the beta-casein sialyl transferase gene in their genome. As shown below, the transgenic goats produce sialyl transferase in their milk.

Sialyl transferase is produced in the mammary gland ("in vitro" activity)

The presence of sialyl transferase in the mammary gland of the transgenic goats was confirmed using an assay to test for enzymatic activity of sialyl transferase. Namely, sialyl transferase can sialylate exposed 1,4 galactose on N-linked complex sugars.

Recombinant Alpha-1 antitrypsin, (AAT) when produced in the milk of transgenic goats is 50% sialylated, whereas AAT isolated from serum is almost completely sialylated (*e.g.,* the commercially available Alfalastin®). The recombinant Alpha-1 antitrypsin produced in the milk of transgenic goats was used as a target for the sialylation reaction with sialyl transferase produced in the milk of transgenic goats. Milk from goats that express sialyl transferase in the mammary gland, along with nucleotide sugar CMP-sialic acid, was incubated with the undersialylated AAT. In addition, commercially available sialyl transferase was used as a control. The addition of sialic acid to the undersialylated AAT upon incubation was confirmed by running the incubated AAT on an isoelectric focusing gel (IEF gel). Increased levels of sialic acid on the protein (AAT) result in a lower isoelectronic point (pI) causing the protein to run lower on the gel. The recombinant AAT (which is 50% sialylated) is expected to run higher on the gel than the positive control, the fully sialylated AAT Alfalastin® isolated from serum.

The left panel of Figure 3A shows a sialylation experiment comparing AAT (lane 2) and undersialylated AAT that was treated with a mix of commercially available sialyl transferases (lane 3). As expected, the AAT that was subjected to sialyl transferases (lane 3) ran lower on the IEF gel than AAT that was not subjected to sialyl transferases (lane 2).

Similar results are depicted in the right panel of Figure 3A. Treatment of undersialylated AAT with a sialyl transferase mix (lane 3) or purified batches of sialyl transferases from milk (lanes 4 and 5) resulted in increased levels of sialic acid compared and a downward shift compared to untreated undersialylated AAT (lane 2).

Figure 3B depicts a gel showing Alfalastin®, a sialylated version of AAT (lane 1), AAT treated with milk from sialyl transferase producing transgenic goats (lane 2 and lane 7), AAT treated with milk from a control animal (lanes 3 and 8), AAT treated with milk from a control animal and transgenically produced sialyl transferase (lane 4), AAT treated with buffer and a transgenically produced sialyl transferase (lane 5), and AAT treated with buffer (lane 6).

Thus, as shown in Figure 3, the sialyl transferase ST6Gal1 produced in the milk of transgenic goats is active "*in vitro*" and can sialylate undersialylated AAT to the level of plasma derived fully sialylated AAT.

Glycosylation pattern of AAT treated with transgenically produced sialyl transferase.

The transgenically produced sialyl transferase was purified from the milk of the transgenic animal and used to sialylate the target protein: undersialylated AAT. After subjecting the undersialylated AAT to sialyl transferase, the glycosylation of AAT was evaluated to confirm if sialic acid was added to the exposed Gal residues of the glycosylation side chains of AAT.

As shown in Figure 4, "*in vitro*" sialylation with sialyl transferase harvested from the milk of transgenic goats that produce sialyl transferase converts most of the mono-sialylated biantennary complex sugars on AAT to the bisialylated form. The lower graph shows AAT prior to sialylation while the upper graph shows AAT after being subjected to sialylation.

Sialyl transferase activity in the mammary gland ("in vivo")

Sialyl transferase that is transgenically produced in the mammary gland was evaluated for its ability to increase the level of sialylation of recombinant proteins also being secreted by the mammary gland.

To test if the ST6Gal1 was active in the mammary gland, an endogenous protein was evaluated for the level of sialylation. Lactoferrin is an endogenous milk-produced protein that carries three N-linked glycosylation sites. Lactoferrin was isolated from the milk of a goat that carries the ST6Gal1 gene and produces sialyl transferase in its milk (Figure 5, column 2). Lactoferrin was also isolated from control animal, which does not carry the ST6Gal1 gene (Figure 5, column 1). The two lactoferrin samples, along with duplicate preparations, were treated with N-glycanase, derivatized with 2-aminobenzoic acid (2-AA), and the glycosylation pattern analyzed by HILIC LC-MS/(MS) with fluorescence detection. The results (Figure 5) show that there is increased sialyl transferase activity in the transgenic mammal because the amount of bisialylated N-linked sugars on the lactoferrin is increased from 25% to 34% of the total sugar. The increase in bisialylation is correlated with the conversion of monosialylated sugars, which show a decrease from 18% in the control to 5% in the sialyl transferase expressing goat. Thus, the ST6Gal1 produced in the mammary gland converts most of the undersialylated mono-sialylated N-linked sugar to the bi-sialylated form. The ST6 Gal1 sialyl transferase, therefore, is active in the mammary gland and is capable of adding a sialic acid to a glycoprotein in the mammary gland.

Sialylation of transgenically produced protein

Increasing expression of sialyl transferase in the mammary gland should result in an increase in sialylation of a recombinant protein also produced in the mammary gland. As shown above, ST6Gal1, when secreted into the milk, is active on AAT "*in vitro*". AAT was also used to evaluate the ability of transgenically expressed sialyl transferase to sialylate a transgenically expressed protein. Both ST6 Gal1 and AAT were co-expressed in the same mammary gland. This was achieved by crossing lines of goats carrying AAT with goats carrying the ST6 Gal1, resulting in mammals carrying both constructs. A male goat carrying the gene construct for expression of AAT into the milk (BC30) was crossed with a super-ovulated ST6Gal1 female. Embryos from this cross were isolated and implanted into foster mothers. From this procedure, 26 animals were born. Upon genetic testing, 2 females were identified that carried both the AAT and ST6 Gal1 gene constructs. In addition, 4 females carrying only the AAT construct were identified, providing a negative control.

At 3 months of age the animals that carry both the ST6Gal and AAT genes (and the control animals) were induced to lactate. The AAT was isolated from the animal carrying the ST6Gal1 as well as the control animals. The protein was analyzed with regard to the glycosylation, including the sialic acid content. As shown in Figure 6, the bi-antennary N linked sugars on the control AAT animal are 50% bisialylated (bottom graph). However, the AAT isolated from the line carrying the ST6Gal1 construct was completely sialylated. The increased level of sialic acid on the AAT indicates the ability of the over expressed sialyl transferase to enhance sialylation of mammary gland expressed recombinant proteins.

Example 2

Determining the level of 2,3 sialyl transferase in the mammary gland

The gene for 2,3 sialyl transferase is isolated from a cDNA library from goat liver. This cDNA library is used as a probe in a Northern blot to determine the level of expression of the gene in the mammary gland vs. the liver. A decrease in expression of the 2,3 sialyl transferase in the mammary gland compared to the liver could imply a limitation in the 2,3 sialylation capacity in the mammary gland.

Cloning of ST3 Gal1 from goat liver

A cDNA library is constructed using RNA isolated from goat liver. The sequence of human ST3 is used to design PCR primers that can amplify the goat sequence. Following RT-PCR reactions, a full-length cDNA encoding 2,3 sialyl transferase, (ST3 Gal1), is

isolated. The gene encoding the 2,3 sialyl transferase is ligated into the goat beta casein expression vector to yield a plasmid, such as BC2541 ST3Gal1, including the goat beta-casein promoter to direct expression of linked genes in the mammary gland.

Generation of transgenic goats

An ST3Gal1-containing transgene is used to generate transgenic goats that express the ST3Gal1 in their mammary gland. The resulting goats carry the beta-casein sialyl transferase gene in their genome and produce sialyl transferase in their milk.

Production of sialyl transferase in the mammary gland ("in vitro" activity)

The presence of sialyl transferase in the mammary gland of the transgenic goats is determined using an assay to test for enzymatic activity of sialyl transferase. For example, sialyl transferase can sialylate exposed 1,4 galactose on N-linked complex sugars.

Recombinant Alpha-1 antitrypsin that is produced in the milk of transgenic goats is used as a target for the sialylation reaction with 2,3 sialyl transferase that is produced in the milk of transgenic goats. Milk from goats that express sialyl transferase in the mammary gland, along with nucleotide sugar CMP-sialic acid, is incubated with the undersialylated AAT. In addition, commercially available sialyl transferase is used as a control. The addition of sialic acid to the undersialylated AAT upon incubation is confirmed by running the incubated AAT on an isoelectric focusing gel (IEF gel). Increased levels of sialic acid on the protein (AAT) could result in a lower isoelectronic point (pI) causing the protein to run lower on the gel. The recombinant AAT (which is 50% sialylated) is expected to run higher on the gel than the positive control, the fully sialylated AAT Alfalastin® isolated from serum.

Glycosylation pattern of AAT treated with transgenically produced ST3.

The transgenically produced 2,3 sialyl transferase is purified from the milk of the transgenic animal and used to sialylate the undersialylated AAT. After subjecting the undersialylated AAT to sialyl transferase, the glycosylation of AAT is evaluated to confirm whether sialic acid has been added to the exposed Gal residues of the glycosylation side chains of AAT.

Sialyl transferase activity in the mammary gland ("in vivo")

The 2,3 sialyl transferase that is transgenically produced in the mammary gland is evaluated for its ability to increase the level of sialylation of recombinant proteins also being

secreted by the mammary gland or endogenous proteins secreted by the mammary gland (*e.g.*, lactoferrin).

Sialylation of transgenically produced protein

Increasing expression of sialyl transferase in the mammary gland could result in an increase in sialylation of a recombinant protein also produced in the mammary gland. AAT is used to evaluate the ability of transgenically expressed sialyl transferase to sialylate a transgenically expressed protein. Both ST3 Gal1 and AAT are co-expressed in the same mammary gland. This is achieved by crossing lines of goats carrying AAT with goats carrying the ST3 Gal1, resulting in mammals carrying both constructs. A male goat carrying the gene construct for expression of AAT into the milk (BC30) is crossed with a super-ovulated ST6Gal1 female. Embryos from this cross are isolated and implanted into foster mothers. Offspring are tested and those that carry both the AAT and ST3 Gal1 gene constructs are identified. Other offspring that carry only the AAT construct can be used as a negative control.

At 3 months of age the animals that carry both the ST3Gal and AAT genes (and the control animals) are induced to lactate. The AAT is isolated from the animals carrying the ST3Gal1 as well as the control animals, and the protein can be analyzed with regard to the glycosylation, including the sialic acid content.

Example 3: Pharmacokinetic profiles

Pharmacokinetic profiles of transgenically produced AAT that has been sialylated by ST3 or ST6 was compared to plasma-derived AAT (pdAAT) by analysis of the evolution of the concentration of AAT over a time period of 25 hours, as presented in Figure 7.

Figure 7 shows that AAT sialylated by ST3 or ST6 has a better half-life than no-sialylated AAT (pdAAT).

Equivalents

The foregoing written specification is considered to be sufficient to enable one skilled in the art to practice the invention. The present invention is not to be limited in scope by examples provided, since the examples are intended as an illustration of certain aspects and embodiments of the invention. Other functionally equivalent embodiments are within the scope of 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 advantages and objects of the invention are not necessarily encompassed by each embodiment of the invention.

What is claimed is:

CLAIMS

1. A method of producing protein with modified glycosylation, the method comprising:

providing a transgenic mammal that has been modified to have increased expression of sialyl transferase in the mammary gland,

wherein the transgenic mammal expresses a protein in the mammary gland, and

harvesting the protein from the mammary gland of the transgenic mammal.

2. A method of producing protein with modified glycosylation, the method comprising:

providing a mammary epithelial cell that has been modified to have increased expression of sialyl transferase,

wherein the mammary epithelial cell expresses a protein, and

harvesting the protein from the mammary epithelial cells.

3. The method of claim 1 or claim 2, wherein the glycosylation is modified compared to a protein produced in a transgenic mammal or mammary epithelial cell that has not been modified to have increased expression of sialyl transferase.

4. The method of any one of claims 1-3, wherein the modified glycosylation is increased sialylation.

5. A method of producing protein with increased sialylation, the method comprising:

providing a transgenic mammal that has been modified to have increased expression of sialyl transferase in the mammary gland,

wherein the transgenic mammal expresses a protein in the mammary gland, and

harvesting the protein with increased sialylation from the mammary gland of the transgenic mammal.

6. The method of claim 4 or claim 5, wherein the sialylation is increased compared to a protein produced in a transgenic mammal or mammary epithelial cell that has not been modified to have increased expression of sialyl transferase.

7. The method of any one of claims 1-6, wherein the protein with modified glycosylation or protein with increased sialylation is a human protein.

8. The method of any one of claims 1-7, wherein the protein with modified glycosylation or protein with increased sialylation is an antibody.

9. The method of any one of claims 1-7, wherein the protein with modified glycosylation or protein with increased sialylation is antithrombin.

10. The method of any one of claims 1-7, wherein the protein with modified glycosylation or protein with increased sialylation is alpha-1 antitrypsin.

11. The protein with modified glycosylation or protein with increased sialylation produced according to any of the methods according to claims 1-10.

12. A composition comprising the protein with modified glycosylation or protein with increased sialylation of claim 11, further comprising milk.

13. A method of increasing the sialyl transferase activity in the mammary gland, the method comprising:

providing a transgenic mammal that has been modified to have increased expression of sialyl transferase in the mammary gland.

14. A method of producing sialyl transferase, the method comprising:

providing a transgenic mammal that has been modified to have increased expression of sialyl transferase in the mammary gland, and

harvesting the sialyl transferase from the mammary gland of the transgenic mammal.

15. The method of claim 14, further comprising using the sialyl transferase to sialylate a protein.

16. The method of claim 15, wherein the sialyl transferase is placed in contact with the protein.

17. The sialyl transferase produced according to the method of claim 15 or 16.

18. A composition comprising the sialyl transferase of claim 17, further comprising milk.

19. The method of any one of the preceding claims, wherein the transgenic mammal or mammary gland epithelial cell has been modified by introducing into the genome of the transgenic mammal or mammary gland epithelial cell a nucleic acid sequence encoding a sialyl transferase.

20. The method of any one of the preceding claims, wherein the protein expressed in the transgenic mammal or mammary gland epithelial cell is encoded by a nucleic acid sequence introduced into the genome of the transgenic mammal or mammary gland epithelial cell.

21. A transgenic mammal that has been modified to have increased expression of sialyl transferase in the mammary gland.

22. The transgenic mammal of claim 21, wherein the transgenic mammal further expresses an exogenous protein in the mammary gland.

23. The transgenic mammal of claim 22, wherein the exogenous protein is a human protein.

24. The transgenic mammal of claim 22 or claim 23, wherein the exogenous protein is an antibody.

25. The transgenic mammal of claim 22 or claim 23, wherein the exogenous protein is antithrombin.

26. The transgenic mammal of claim 22 or claim 23, wherein the exogenous protein is alpha-1 antitrypsin.

27. A mammary epithelial cell that has been modified to have increased expression of sialyl transferase.

28. The mammary epithelial cell of claim 27, wherein the mammary epithelial cell further expresses an exogenous protein.

29. The mammary epithelial cell of claim 28, wherein the exogenous protein is a human protein.

30. The mammary epithelial cell of claim 28 or claim 29, wherein the exogenous protein is an antibody.

31. The mammary epithelial cell of claim 28 or claim 29, wherein the exogenous protein is antithrombin.

32. The mammary epithelial cell of claim 28 or claim 29, wherein the exogenous protein is alpha-1 antitrypsin.

33. The method or mammal of any one of the preceding claims, wherein the transgenic mammal is an ungulate.

34. The method or mammal of claim 33, wherein the ungulate is a goat.

35. The method, cell or mammal of any one of the preceding claims, wherein the expression of sialyl transferase and/or the protein is under the control of a milk promoter.

36. The method, cell or mammal of claim 35, wherein the milk promoter is a goat beta casein promoter.

37. The method, cell or mammal of any one of the preceding claims, wherein the sialyl transferase is beta-galactosamide alpha-2,6-sialyltranferase.

38. The method, cell or mammal of any one of the preceding claims, wherein the sialyl transferase is beta-galactosamide alpha-2,3-sialyltranferase.

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Query:   1   MTRTSLKKKFFSSCVLTFLLFAIICVWKEKKNGMYYEFLKLQNKEYQMLQQLEKLAMSSS  60
             M  T+LKKKF SCCVL+FLLFA+ICVWKEKKNG+YY+  KLQ KE+Q+L+ L KLAM S
Sbjct:   1   MIHTMLKKKFLSCCVLAFLLFAVICVWKEKKNGSYYDSFKLQTKEFQVLKSLQSLAMGSD  59

Query:  61   SQPGSSSSTTWMPQKNIQALGG----PKAKLGATFQVWDKDSSSKNLAPRLQTIRKNYLNM  116
             SQ SSSST +P + Q LG     KAK  A+FQVW+KDSSSKNL PRLQ I KNYL+M
Sbjct:  60   SQSVSSSSTNDPHHGRQTLGSLRGLAKAKPEASFQVWNKDSSSKNLIPRLQKIMKNYLSM  119

Query: 117   NKYKVTYKGPGPGVKFSAEALLCHLRDHVNISMIEATDFPFNTSDWEGYLPQEDIRTKAG  176
             NKYKV+YKGPGPG+KFSAEAL CHLRDHVN+SM+E TDFPFNTS+WEGYLP+E IRTKAG
Sbjct: 120   NKYKVSYKGPGPGIKFSAEALRCHLRDHVNVSMVEVTDFPFNTSEWEGYLPKESIRTKAG  179

Query: 177   PWGRCAVVSSAGSLKSSRLGREIDDEDAVLRFNGAPTVKFQQDVGTKTTIRLVNSQLVTM  236
             PWGRCAVVSSAGSLKSS+LGREITDEDAVLRFNGAPT  FQQDVGTKTTIRL+NSQLVTM
Sbjct: 180   PWGRCAVVSSAGSLKSSQLGREITDEDAVLRFNGAPTANFQQDVGTKTTIRLMNSQLVTM  239

Query: 237   EAGFLKDSLYNEGILIVWEPSVYHSDIPKWYRNPDYSFFNNFKSYRKLHPDQFEYILKFQ  296
             E  FLKDSLYNEGILIVWEPSVYHSDIPKWYQNPDYNFFNNYKSYRKLHPNQFEYILKFQ
Sbjct: 240   EKRFLKDSLYNEGILIVWEPSVYHSDIPKWYQNPDYNFFNNYKSYRKLHPNQFEYILKFQ  299

Query: 297   MPWELMDIIQEISSKELTQPNPFSSGMLGIAIMMSLCDQVDIYEFLPSKRKTDVCYYQRAY  356
             MPWELMDI+QEIS E TQPNPFSSGMLGI IMM+LCDQVDIYEFLPSKRKTDVCYYQ++
Sbjct: 300   MPWELMDILQEISPEETQPNPFSSGMLGIIIMMTLCDQVDIYEFLPSKRKTDVCYYQKF  359

Query: 357   FDSACTMGAYHPILYEKMVKHLRLGTDEDIYLLGKAMLPGFRTIRC 403
             FDSACTMGAYHPILYEKMVKHLRLGTDEDIYLLGKAMLPGFRTIRC
Sbjct: 360   FDSACTMGAYHPILYEKMLVKHLRQGTDEDIYLLGKAMLPGFRTIHC 406
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FIGURE 1

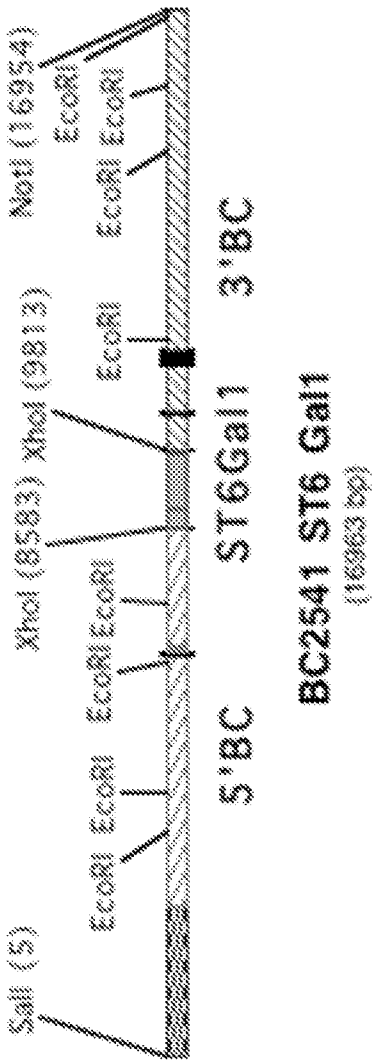
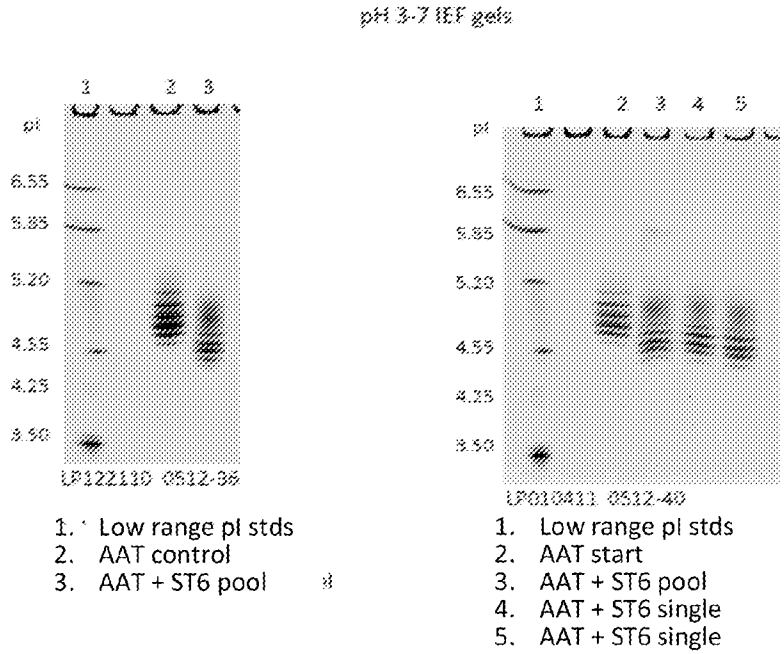

SalI (5)
EcoRI
EcoRI
EcoRI
EcoRI
XhoI (8583)
XhoI (9813)
EcoRI
EcoRI
EcoRI
NotI (16954)
5'BC
ST6Gal1
3'BC
BC2541 ST6 Gal1
(16963 bp)

FIGURE 2

A


pH 3-7 IEF gels
1 2 3
pI
6.55
5.85
5.20
4.55
4.25
3.50
LP122130 0512-36
1. Low range pI stds
2. AAT control
3. AAT + ST6 pool
1 2 3 4 5
pI
6.55
5.85
5.20
4.55
4.25
3.50
LP010411 0512-40
1. Low range pI stds
2. AAT start
3. AAT + ST6 pool
4. AAT + ST6 single
5. AAT + ST6 single

B

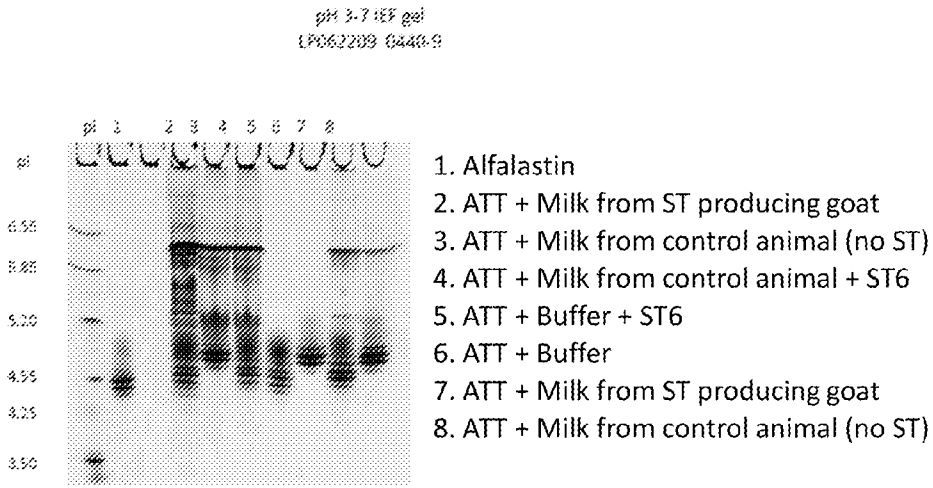

pH 3-7 IEF gel
LP062209 0440-9
pI 1 2 3 4 5 6 7 8
pI
6.55
5.85
5.20
4.55
4.25
3.50
1. Alfalastin
2. ATT + Milk from ST producing goat
3. ATT + Milk from control animal (no ST)
4. ATT + Milk from control animal + ST6
5. ATT + Buffer + ST6
6. ATT + Buffer
7. ATT + Milk from ST producing goat
8. ATT + Milk from control animal (no ST)

FIGURE 3

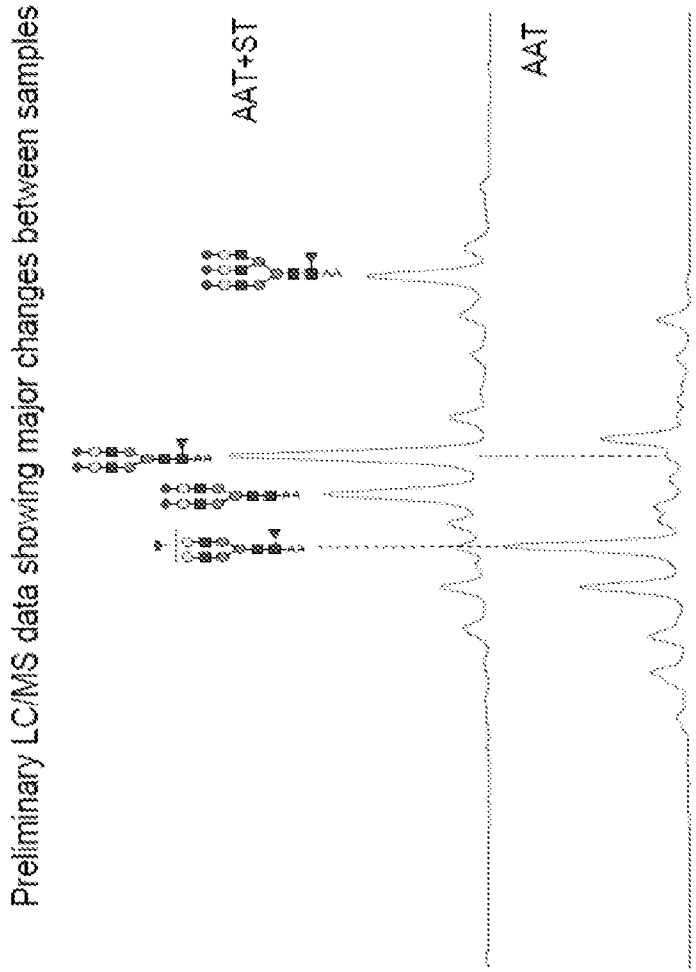

Preliminary LC/MS data showing major changes between samples
AAT+ST
AAT

FIGURE 4

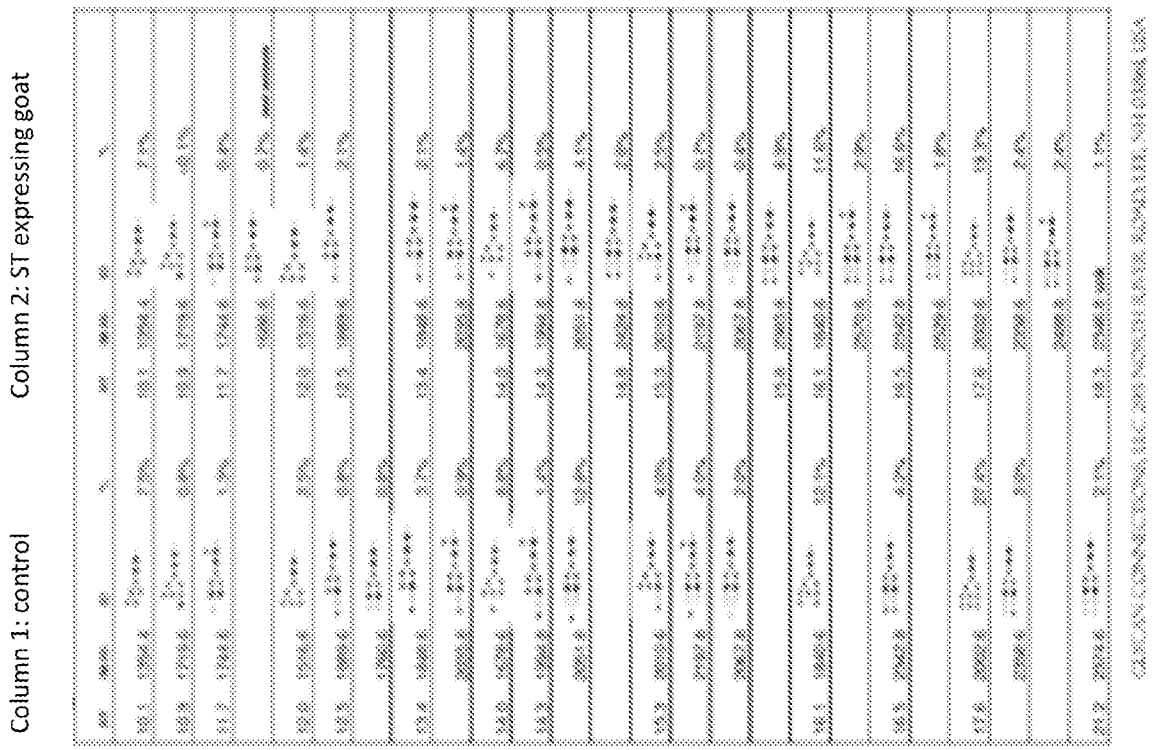


FIGURE 5

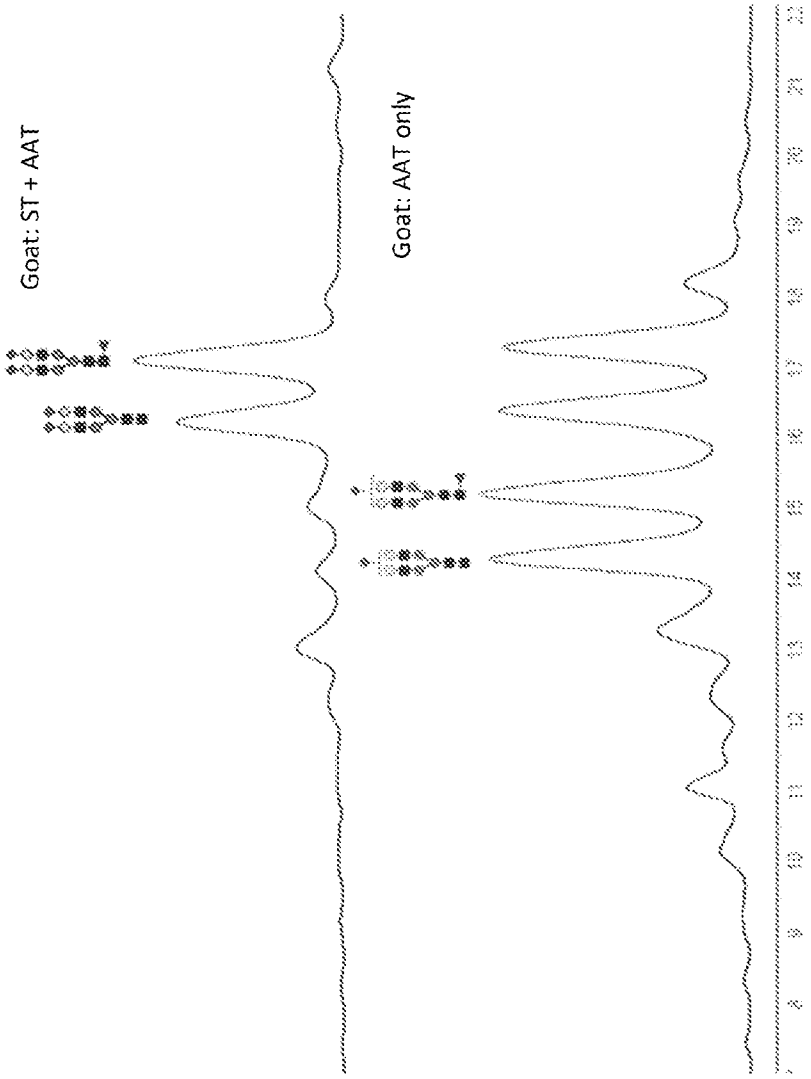


FIGURE 6

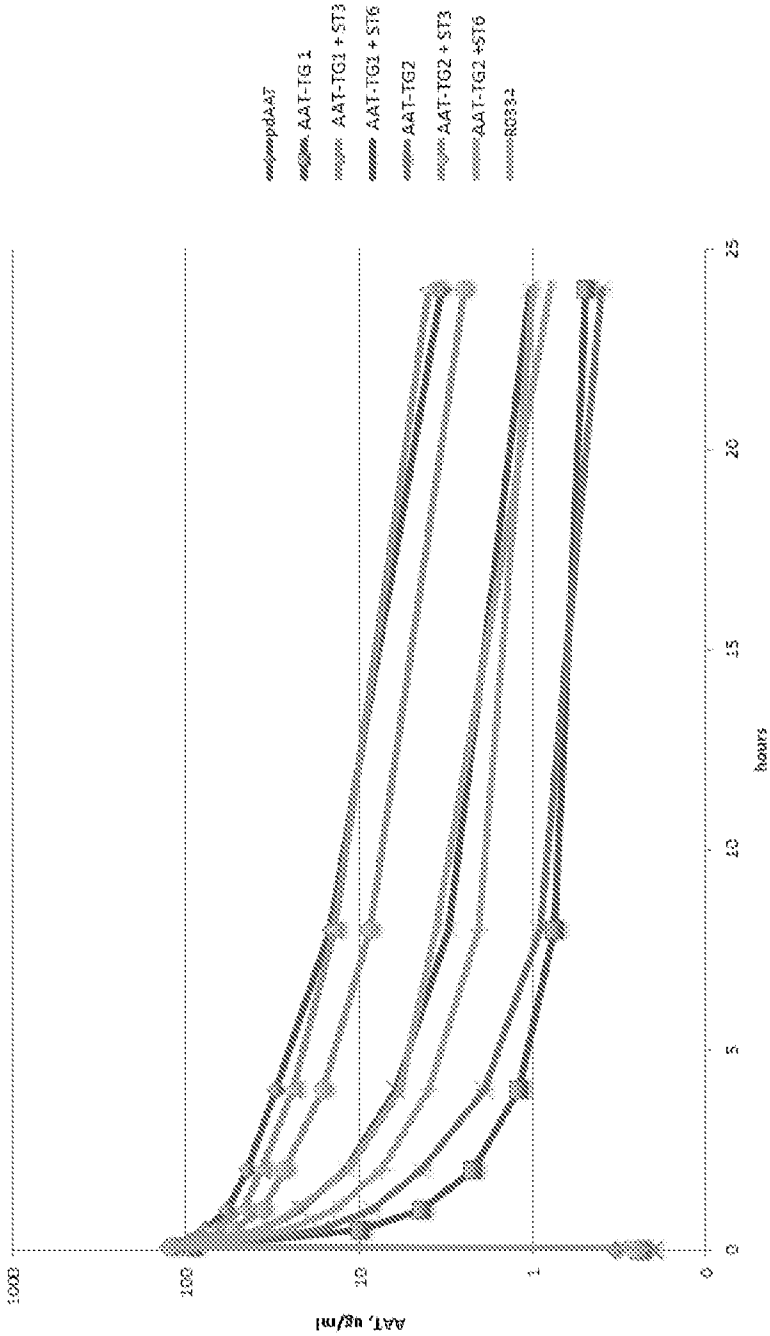

pAAT
AAT-TG 1
AAT-TG1 + ST3
AAT-TG1 + ST6
AAT-TG2
AAT-TG2 + ST3
AAT-TG2 +ST6
R333A
AAT, ug/ml
1000
100
10
1
0
0
5
10
15
20
25
hours

FIGURE 7