



(51) International Patent Classification:

C12N 9/28 (2006.01) C12N 15/62 (2006.01)

C12N 9/24 (2006.01) C07K 16/00 (2006.01)

C07K 14/195 (2006.01)

(21) International Application Number:

PCT/US2020/045054

(22) International Filing Date:

05 August 2020 (05.08.2020)

(25) Filing Language:

English

(26) Publication Language:

English

(30) Priority Data:

62/882,729 05 August 2019 (05.08.2019) US

(71) Applicant: CHO PHARMA, INC.; 7F., Building C, No. 99, Ln. 130, Sec. 1, Academia Rd Nangang Dist., Taipei City, 115202 (TW).

(71) Applicant (for MW only): CHO PHARMA USA, INC. [US/US]; 184 E. Emerson Rd., Lexington, MA 02420 (US).

(72) Inventors: CHU, Kuo-Ching; 7F., Building C, No. 99, Ln. 130, Sec. 1, Academia Rd., Nangang Dist., Taipei City,

115202 (TW). HUANG, Lin-Ya; 7F., Building C, No. 99, Ln. 130, Sec. 1, Academia Rd., Nangang Dist., Taipei City, 115202 (TW). ZENG, Yi-Fang; 7F., Building C, No. 99, Ln. 130, Sec. 1, Academia Rd., Nangang Dist., Taipei City, 115202 (TW).

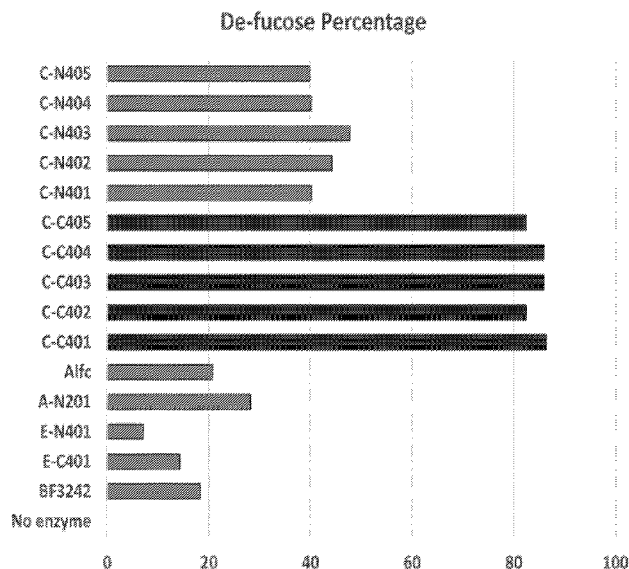
(74) Agent: YANG, Kay; Prosyla Group, PC, 4275 Executive Square, Suite 200, La Jolla, CA 92037 (US).

(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, DJ, DK, DM, DO, DZ, EC, EE, EG, ES, FI, GB, GD, GE, GH, GM, GT, HN, HR, HU, ID, IL, IN, IR, IS, IT, JO, JP, KE, KG, KH, KN, KP, KR, KW, KZ, LA, LC, LK, LR, LS, 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, ST, SV, SY, TH, TJ, TM, TN, TR, TT, TZ, UA, UG, US, UZ, VC, VN, WS, 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, ST, SZ, TZ,

(54) Title: FUSION PROTEIN FOR REMODELING ANTIBODY GLYCOFORM

FIG. 1



(57) Abstract: The present disclosure provides a fusion protein comprising a fucosidase or a truncated fragment or a mutant thereof fuses with either N-terminal end or C-terminal end of the endoglycosidase or a truncated fragment of mutant thereof. The present disclosure also provides a nucleic acid molecule expressing the fusion protein and a method for remodeling a glycan of an antibody Fc region.



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:

- *with international search report (Art. 21(3))*
- *with sequence listing part of description (Rule 5.2(a))*

FUSION PROTEIN FOR REMODELING ANTIBODY GLYCOFORM

[0001] This application claims the benefit and priority to U.S. Provisional Application No. 62/882,729, filed on August 5, 2019, entitled, “Advanced Fucosidase for Cleaving Core-Fucose of Ab-Fc”, the contents of which is incorporated by reference herewith in its entirety.

5

SEQUENCE LISTING

[0002] This instant application contains a Sequence Listing which has been submitted electronically in ASCII format and is hereby incorporated by reference in its entirety. Said ASCII copy, created on August 5, 2020, is named G4590-09600PCT_SeqList.txt and is 1.5 MB in size.

FIELD OF THE DISCLOSURE

10 [0003] The present disclosure relates to glycoform remodeling; particularly, to a fusion protein for remodeling antibody glycoform.

BACKGROUND OF THE DISCLOSURE

[0004] Antibodies, also known as immunoglobulins (Ig), are glycoproteins that play a central role in immune responses. IgG antibodies and fragments of IgG antibodies have been developed as important bio-therapeutics for treating a diverse range of diseases. The constant (Fc) region of naturally-occurring antibodies is typically N-glycosylated. The particular glycosylation, i.e., glycoform, significantly affects Fc effector functions and stability of the antibodies. Thus, one approach to improve therapeutic efficacy of an antibody is to modify the glycoform in the Fc region.

20 [0005] The Fc glycoform typically includes a core fucose residue. In the case of cancer therapy, it has been reported that anti-tumor antibodies having glycoforms in the Fc region with lower levels of core fucose demonstrate higher binding affinity to CD16 (FcγRIIIA) on NK cells. The higher affinity results in an improved induction of antibody-dependent cellular cytotoxicity against tumor cells.

25 [0006] To produce antibodies with a low level of core fucose, several strategies have been developed. For example, an inhibitor of fucosyltransferase, which is necessary for core fucose addition, can be added to the culture medium used for growing antibody producing cells. In another example, antibody producing cells can be genetically engineered to lack fucosyltransferase activity.

30 [0007] In a different strategy, purified enzymes can be used *in vitro* to directly modify the antibody glycoform in a predictable way. One advantage of this strategy is that antibodies can be produced and then modified to have well-defined low core fucose Fc glycoforms with desirable biological properties. This would facilitate the use of high-yielding non-mammalian expression systems to produce antibodies instead of mammalian expression systems, which are

typically employed to ensure proper core glycosylation. US 20190185898 provides for fucosidase mutants that serve as fuco-ligases for core fucosylation of a range of biological glycopeptides and glycoproteins including intact therapeutic antibodies.

[0008] Yet, existing fucosidase enzymes are inefficient at cleaving core fucose from the Fc region. The need exists to develop enzymes that can efficiently and predictably remodel antibody glycoform.

SUMMARY OF THE DISCLOSURE

[0009] A fusion protein is provided in the disclosure. The assembly of multiple enzymes in a fusion protein facilitates enzyme synergism and exhibits advantageous effect of remodeling antibody glycoform predictably.

[0010] In one aspect, the present disclosure provides a fusion protein comprising a fucosidase or a truncated fragment or a mutant thereof fuses with either N-terminal end or C-terminal end of the endoglycosidase or a truncated fragment or a mutant thereof, wherein the fusion protein exhibit both the fucosidase activity and the endoglycosidase activity.

[0011] Certain embodiments of the fucosidase include, but are not limited to, *Lactobacillus casei* α -L fucosidase C (Alfc), *Bacteroides fragilis* fucosidase (BF3242), *Bacteroides thetaiotamicron* α -L-fucosidase (BT2970), *Emticicia oligotrophica* α -L-fucosidase (EO0918) and *Elizabethkingia miricola* α -(1-6) fucosidase (Emfuc3), or a fragment thereof, or a mutant thereof. In some embodiments, the fucosidase is *Lactobacillus casei* α -L fucosidase C, *Elizabethkingia miricola* α -(1-6) fucosidase (Emfuc3), or a truncated fragment thereof, or a mutant thereof.

[0012] Certain embodiments of the endoglycosidase include, but are not limited to, *Streptococcus pyogenes* endoglycosidase S, *Streptococcus pyogenes* endoglycosidase S2 or a fragment or a mutant thereof. Particularly, the endoglycosidase is *Streptococcus pyogenes* endoglycosidase S2, or a truncated fragment or a mutant thereof.

[0013] In one embodiment, the fucosidase or endoglycosidase is a truncated fragment. Truncated fragment of endoglycosidase is the IgG binding domain thereof. In some embodiments, the truncated fragment of the endoglycosidase is an IgG binding domain of *Streptococcus pyogenes* endoglycosidase S or *Streptococcus pyogenes* endoglycosidase S2.

[0014] In one embodiment, the fucosidase or endoglycosidase is a mutant polypeptide. Particularly, the endoglycosidase mutant is *Streptococcus pyogenes* endoglycosidase S having a mutation at amino acid position -D233, preferably -D233Q. In some embodiments, the endoglycosidase mutant is *Streptococcus pyogenes* endoglycosidase S2 having a mutation at amino acid position T138, D182, D184, D186, D226, or T227; preferably having a T138E, T138M, T138Q, T138R, T138M, T138L, T138H, T138N, T138K, D182Q, D184M, D184Q, D184T, D184L, D184F, D184S, D184V, D184K, D184W, E186A, D226Q, or T227Q.

[0015] Certain embodiments of the fusion protein include, but are not limited to, *Lactobacillus casei* α -L fucosidase C fused with *Streptococcus pyogenes* endoglycosidase S, *Streptococcus pyogenes* endoglycosidase S2, an IgG binding domain of *Streptococcus pyogenes* endoglycosidase S, an IgG binding domain of *Streptococcus pyogenes* endoglycosidase S2, *Streptococcus pyogenes* endoglycosidase S having a mutation at amino acid position D233, *Streptococcus pyogenes* endoglycosidase S2 having a mutation at amino acid position T138, *Streptococcus pyogenes* endoglycosidase S2 having a mutation at amino acid position D182, *Streptococcus pyogenes* endoglycosidase S2 having a mutation at amino acid position D184, *Streptococcus pyogenes* endoglycosidase S2 having a mutation at amino acid position D186, *Streptococcus pyogenes* endoglycosidase S2 having a mutation at amino acid position D226, or *Streptococcus pyogenes* endoglycosidase S2 having a mutation at amino acid position T227.

[0016] Particularly, the fusion protein comprises an amino acid sequence selected from the group consisting of: SEQ ID NOs: 2, 4, 6, 8, 10, 12, 14, 16, 18, 20, 22, 24, 26, 28, 30, 32, 34, 36, 38, 40, 42, 44, 46, 48, 50, 52, 54, 56, 58, 60, 62, 64, 66, 68, 70, 72, 74, 76, 78, 80, 82, 84, 86, 88, 90, 92, 94, 96, 98, 100, 102, 104, 106, 108, 110, 112, 114, 116, 118, 120, 122, 124, 126, 128, 130, 132, 134, 136, 138, 140, 142, 144, 146, 148, 150, 152, 154, 156, 158, 160, 162, 164, 166, 168, 170, 172, 174, 176, 178, 180, 182, 184, 186, 188, 190, 192, 194, 196, 198, 200, 202, and 204, or a substantially similar sequence thereof. Preferably, the fusion protein has the amino acid sequence selected from the group consisting of SEQ ID NOs: 34, 36, 38, 40, 42, 44, 46, 48, 50, 52, 54, 56, 58, 60, 62, 64, 66, 68, 70, 72, 74, 76, 78, 80, 82, 84, 86, 88, 90, 92, 94, 96, 98, 100, 102, 104, 106, 108, 174, 176, 178, 180, 182, 184, 186, 188, 190, 192, 194, 196, 198, 200, 202, and 204, or a substantially similar sequence thereof.

[0017] In another aspect, the present disclosure provides a nucleic acid molecule expressing the fusion protein as described herein. Certain embodiments of the nucleic acid include, but are not limited to, SEQ ID NOs: 1, 3, 5, 7, 9, 11, 13, 15, 17, 19, 21, 23, 25, 27, 29, 31, 33, 35, 37, 39, 41, 43, 45, 47, 49, 51, 53, 55, 57, 59, 61, 63, 65, 67, 69, 71, 73, 75, 77, 79, 81, 83, 85, 87, 89, 91, 93, 95, 97, 99, 101, 103, 105, 107, 109, 111, 113, 115, 117, 119, 121, 123, 125, 127, 129, 131, 133, 135, 137, 139, 141, 143, 145, 147, 149, 151, 153, 155, 157, 159, 161, 163, 165, 167, 169, 171, 173, 175, 177, 179, 181, 183, 185, 187, 189, 191, 193, 195, 197, 199, 201, and 203, or a substantially identical sequence thereof. In some further embodiments, the nucleic acid has the nucleotide sequence selected from the group consisting of SEQ ID NOs: 33, 35, 37, 39, 41, 43, 45, 47, 49, 51, 53, 55, 57, 59, 61, 63, 65, 67, 69, 71, 73, 75, 77, 79, 81, 83, 85, 87, 89, 91, 93, 95, 97, 99, 101, 103, 105, 107, 173, 175, 177, 179, 181, 183, 185, 187, 189, 191, 193, 195, 197, 199, 201, and 203, or a substantial identical sequence thereof.

[0018] In another aspect, the present disclosure provides a vector comprising the nucleic acid of the present disclosure. The present disclosure also provides a host cell comprising the vector.

[0019] In a further aspect, the present disclosure provides a method for remodeling a glycan of a Fc region of an antibody comprising the following steps: obtaining an antibody that has a heterogeneous glycan in the Fc region of the antibody and contacting the antibody with the fusion protein as described herein. Particularly, the method comprises providing an antibody having a heterogeneous glycan in the Fc region thereof, a fusion protein as described herein and a target glycan oxazoline, wherein the glycan of the antibody Fc region comprises a N-acetylglucosamine (GlcNAc) residue; and contacting the antibody with the fusion protein and the target glycan oxazoline linking to the antibody Fc region; whereby a remodeled glycan of the antibody Fc region can be obtained.

[0020] In one embodiment of the disclosure, the method further comprises purifying the antibody having the remodeled glycan in the Fc region.

[0021] The present disclosure is described in detail in the following sections. Other characteristics, purposes and advantages of the present disclosure can be found in the detailed description and claims.

BRIEF DESCRIPTION OF THE DRAWINGS

[0022] FIG. 1 shows a bar graph showing percentage of fucose hydrolysis by the indicated enzymes, i.e., fucosidases, and fucosidase/EndoS2 fusion proteins, expressed as percentage of starting fucose removed. The enzymes are identified in Tables 1 to 5 below.

[0023] FIG. 2 shows a bar graph showing percentage of fucose hydrolysis by Alfc fucosidase or Alfc/EndoS2 fusion proteins expressed as percentage of starting fucose removed. The fusion proteins are identified in Tables 1 to 5 below.

[0024] FIG. 3 shows a bar graph showing percentage of fucose hydrolysis by Alfc fucosidase or fusion proteins of five fucosidases with truncated EndoS2 expressed as percentage of starting fucose removed. The fusion proteins are identified in Tables 1 to 5 below.

[0025] FIG. 4 shows a bar graph showing percentage of fucose hydrolysis by Alfc or Emfuc3 fucosidase or Alfc or Emfuc3/EndoS2 fusion proteins expressed as percentage of starting fucose removed. The fusion proteins are identified in Tables 1 to 5 below.

[0026] FIG. 5 shows a bar graph showing percentage of fucose hydrolysis by Alfc or Emfuc3 fucosidase, Alfc/EndoS or EndoS or EndoS2/Emfuc3 fusion proteins expressed as percentage of starting fucose removed. The fusion proteins are identified in Tables 1 to 5 below.

[0027] FIG. 6 shows a bar graph showing percentage of fucose hydrolysis by Alfc, Emfuc3 or BF3242 fucosidase, EndoS or EndoS2/Emfuc3 or EndoS/BF3242 fusion proteins expressed

as percentage of starting fucose removed. The fusion proteins are identified in Tables 1 to 5 below.

[0028] FIG. 7 shows a bar graph showing percentage of fucose hydrolysis by BF3242 fucosidase, EndoS or EndoS2/BF3242 fusion proteins expressed as percentage of starting fucose removed. The fusion proteins are identified in Tables 1 to 5 below.

[0029] FIG. 8A shows SDS-PAGE analysis of the time course of glycan remodeling of trastuzumab (TRZ) by C-C401. The two native glycans are removed and each replaced by Sia₂(α 2-6)Gal₂GlcNAc₂Man₃GlcNAc₂ (G2S2). Lane 2: Standard of TRZ. Lane 3: Standard of TRZ-N/N (TRZ containing two GlcNAc). Lane 4: Treating TRZ with C-C401 for 20 hours to obtain TRZ-N/N. Lanes 5-7: 95%, 98%, and 98% glycosylation of TRZ-N/N with SCT-oxazoline by C-C401 for 10, 20, and 30 minutes, respectively. Lane 8: Purified glyco-engineered TRZ (TRZ-G2S2/G2S2).

[0030] FIG. 8B shows SDS-PAGE analysis of glycan remodeling of TRZ by C-C401. The two native glycans are removed and each replaced by Gal₂GlcNAc₂Man₃GlcNAc₂ (G2). Lane 2: Standard of trastuzumab. Lane 3: Treating TRZ with C-C401 for 16 hours to obtain TRZ-N/N. Lanes 4-7: 98% glycosylation of TRZ-N/N with CT-oxazoline by C-C401 for 15, 30, 45, and 60 minutes. Lane 8: Purified glyco-engineered TRZ (TRZ-G2/G2). Lane 9: Standard of TRZ-G2S2/G2S2.

[0031] FIG. 9 shows SDS-PAGE analysis of glycan remodeling of rituximab (RTX) by C-C401. Lane 2: Standard of trastuzumab. Lane 3: Standard of TRZ-N/N. Lane 4: Standard of rituximab. Lane 5: Treating RTX with C-C401 for 20 hours to obtain RTX-N/N. Lanes 6-8: 95%, 98%, and 98% glycosylation of RTX-N/N with SCT-oxazoline by C-C401. Lane 9: Purified glyco-engineered RTX (RTX-G2S2/G2S2).

[0032] FIG. 10 shows SDS-PAGE analysis of glycan remodeling of TRZ by C-C406. Lane 2: Standard of trastuzumab. Lane 3: Standard of TRZ-N/N. Lane 4: Treating TRZ with C-C406 for 16 hours to obtain TRZ-N/N. Lanes 5-8: 90%, 95%, 98%, and 98% glycosylation of TRZ-N/N with SCT-oxazoline by C-C406 for 30, 60, 90, and 120 minutes respectively. Lane 9: Purified glyco-engineered TRZ (TRZ-G2S2/G2S2).

[0033] FIG. 11 shows SDS-PAGE analysis of glycan remodeling of TRZ by C-C407. Lane 2: Standard of trastuzumab. Lane 3: Standard of TRZ-N/N. Lanes 4-7: 80%, 95%, 98%, and 98% glycosylation of TRZ-N/N with SCT-oxazoline by C-C407 for 30, 60, 90, and 120 minutes, respectively. Lane 8: Treating TRZ with C-C407 for 12 hours to obtain TRZ-N/N. Lane 9: Purified glyco-engineered TRZ (TRZ-G2S2/G2S2).

[0034] FIG. 12 shows SDS-PAGE analysis of glycan remodeling of TRZ by C-C204. Lane 2: Standard of trastuzumab. Lane 3: Treating TRZ with C-C204 for 16 hours to obtain TRZ-N/N. Lane 4-9: 85%, 97%, 98%, 98%, 97%, and 90% glycosylation of TRZ-N/N with SCT-

oxazoline by C-C204 for 15, 30, 45, 60, 90, 120, and 240 minutes, respectively. Lane 10: Purified glyco-engineered TRZ (TRZ-G2S2/G2S2).

DETAILED DESCRIPTION OF THE DISCLOSURE

[0035] The Fc domain of all IgGs possesses a conserved N-glycosylation site to which a biantennary complex-type glycan is typically attached, but significant structural heterogeneity arises from terminal and core modifications. Certain glycoforms of a human antibody exhibit improved therapeutic effects while others possess undesired properties. Therefore, the ability to control the glycosylation process plays a crucial role to obtain antibodies comprising desired glycoform(s) so as to improve their therapeutic efficacy. The present disclosure provides a fusion protein comprising a plurality of enzymes for remodeling antibody glycoforms, following which a glycopeptide having a glycosylation pattern suitable for therapeutic use in a mammal can be generated.

[0036] The practice of the present invention will employ, unless otherwise indicated, conventional techniques of molecular biology, microbiology, recombinant DNA, and immunology, which are within the skill of the art. Such techniques are explained fully in the literature. See, for example, Molecular Cloning A Laboratory Manual, 2nd Ed., ed. by Sambrook, Fritsch and Maniatis (Cold Spring Harbor Laboratory Press, 1989); DNA Cloning, Volumes I and II (D. N. Glover ed., 1985); Culture Of Animal Cells (R. I. Freshney, Alan R. Liss, Inc., 1987); Immobilized Cells And Enzymes (IRL Press, 1986); B. Perbal, A Practical Guide To Molecular Cloning (1984); the treatise, Methods In Enzymology (Academic Press, Inc., N.Y.); Gene Transfer Vectors For Mammalian Cells (J. H. Miller and M. P. Calos eds., 1987, Cold Spring Harbor Laboratory); Methods In Enzymology, Vols. 154 and 155 (Wu et al. eds.), Immunochemical Methods In Cell And Molecular Biology (Mayer and Walker, eds., Academic Press, London, 1987); Antibodies: A Laboratory Manual, by Harlow and Lane s (Cold Spring Harbor Laboratory Press, 1988); and Handbook Of Experimental Immunology, Volumes I-IV (D. M. Weir and C. C. Blackwell, eds., 1986).

[0037] Definitions

[0038] The present disclosure can be more readily understood by reference to the following detailed description of various embodiments of the disclosure, the examples, and the drawings and tables with their relevant descriptions. It is to be understood that unless otherwise specifically indicated by the claims, the disclosure is not limited to specific preparation methods, carriers or formulations, or to particular modes of formulating the extract of the disclosure into products or compositions intended for topical, oral or parenteral administration, because as one of ordinary skill in the relevant arts is well aware, such things can, of course, vary. It is also to be understood that the terminology used herein is for the purpose of describing particular embodiments only and is not intended to be limiting.

[0039] As utilized in accordance with the present disclosure, the following terms, unless otherwise indicated, shall be understood to have the following meaning.

[0040] It must be noted that, as used in the specification and the appended claims, the singular forms "a," "an" and "the" include plural referents unless the context clearly dictates otherwise.

5 Thus, unless otherwise required by context, singular terms shall include the plural and plural terms shall include the singular.

[0041] As used herein, the term "optional" or "optionally" means that the subsequently described event or circumstance may or may not occur, and that the description includes instances where the said event or circumstance occurs and instances where it does not. For
10 example, the phrase "optionally comprising an agent" means that the agent may or may not exist.

[0042] As used herein, the term "glycan" refers to a polysaccharide, or oligosaccharide. Glycan is also used herein to refer to the carbohydrate portion of a glycoconjugate, such as a glycoprotein, glycolipid, glycopeptide, glycoproteome, peptidoglycan, lipopolysaccharide or a
15 proteoglycan. Glycans usually consist solely of O-glycosidic linkages between monosaccharides. For example, cellulose is a glycan (or more specifically a glucan) composed of -1,4-linked D-glucose, and chitin is a glycan composed of -1,4-linked N-acetyl-D-glucosamine. Glycans can be homo or heteropolymers of monosaccharide residues, and can be linear or branched. Glycans can be found attached to proteins as in glycoproteins and
20 proteoglycans. They are generally found on the exterior surface of cells. O- and N-linked glycans are very common in eukaryotes but may also be found, although less commonly, in prokaryotes. N-Linked glycans are found attached to the R-group nitrogen (N) of asparagine in the sequon. The sequon is a Asn-X-Ser or Asn-X-Thr sequence, where X is any amino acid except proline.

25 [0043] As used herein, the term "engineered" describes a polypeptide whose amino acid sequence has been designed by man and/or whose existence and production require human intervention and/or activity.

[0044] As used herein, the term "polynucleotide," or "nucleic acid," as used interchangeably herein, refer to polymers of nucleotides of any length, and include DNA and RNA. The
30 nucleotides can be deoxyribonucleotides, ribonucleotides, modified nucleotides or bases, and/or their analogs, or any substrate that can be incorporated into a polymer by DNA or RNA polymerase, or by a synthetic reaction. A polynucleotide may comprise modified nucleotides, such as methylated nucleotides and their analogs. If present, modification to the nucleotide structure may be imparted before or after assembly of the polymer. The sequence of nucleotides
35 may be interrupted by non-nucleotide components. A polynucleotide may be further modified after synthesis, such as by conjugation with a label. Other types of modifications include, for

example, "caps," substitution of one or more of the naturally occurring nucleotides with an analog, internucleotide modifications such as, for example, those with uncharged linkages (e.g., methyl phosphonates, phosphotriesters, phosphoamidates, carbamates, etc.) and with charged linkages (e.g., phosphorothioates, phosphorodithioates, etc.), those containing pendant moieties, such as, for example, proteins (e.g., nucleases, toxins, antibodies, signal peptides, ϵ -L-lysine, etc.), those with intercalators (e.g., acridine, psoralen, etc.), those containing chelators (e.g., metals, radioactive metals, boron, oxidative metals, etc.), those containing alkylators, those with modified linkages (e.g., alpha anomeric nucleic acids, etc.), as well as unmodified forms of the polynucleotides(s). Further, any of the hydroxyl groups ordinarily present in the sugars may be replaced, for example, by phosphonate groups, phosphate groups, protected by standard protecting groups, or activated to prepare additional linkages to additional nucleotides, or may be conjugated to solid or semi-solid supports. The 5' and 3' terminal OH can be phosphorylated or substituted with amines or organic capping group moieties of from 1 to 20 carbon atoms. Other hydroxyls may also be derivatized to standard protecting groups. Polynucleotides can also contain analogous forms of ribose or deoxyribose sugars that are generally known in the art, including, for example, 2'-O-methyl-, 2'-O-allyl, 2'-fluoro- or 2'-azido-ribose, carbocyclic sugar analogs, alpha-anomeric sugars, epimeric sugars such as arabinose, xyloses or lyxoses, pyranose sugars, furanose sugars, sedoheptuloses, acyclic analogs and basic nucleoside analogs such as methyl riboside. One or more phosphodiester linkages may be replaced by alternative linking groups. These alternative linking groups include, but are not limited to, embodiments wherein phosphate is replaced by P(O)S ("thioate"), P(S)S ("dithioate"), "(O)NR₂" ("amidate"), P(O)R, P(O)OR', CO or CH₂ ("formacetal"), in which each R or R' is independently H or substituted or unsubstituted alkyl (1-20 C) optionally containing an ether (--O--) linkage, aryl, alkenyl, cycloalkyl, cycloalkenyl or araldyl. Not all linkages in a polynucleotide need be identical. The preceding description applies to all polynucleotides referred to herein, including RNA and DNA.

[0045] As used herein, the term "vector" refers to a nucleic acid molecule capable of transporting another nucleic acid to which it has been linked.

[0046] As used herein, the term "host" refers to a system (e.g., a cell, organism, etc.) in which a polypeptide of interest is present. In some embodiments, a host is a system that is susceptible to infection with a particular infectious agent. In some embodiments, a host is a system that expresses a particular polypeptide of interest.

[0047] As used herein, the term "amino acid" refers to naturally occurring and synthetic amino acids, as well as amino acid analogs and amino acid mimetics that function in a manner similar to the naturally occurring amino acids. Naturally occurring amino acids are those encoded by the genetic code, as well as those amino acids that are later modified, e.g., hydroxyproline, L-carboxyglutamate, and O-phosphoserine. Amino acid analogs refers to

compounds that have the same basic chemical structure as a naturally occurring amino acid, i.e., an .alpha. carbon that is linked to a hydrogen, a carboxyl group, an amino group, and an R group, e.g., homoserine, norleucine, methionine sulfoxide, methionine methyl sulfonium. Such analogs have modified R groups (e.g., norleucine) or modified peptide backbones, but retain the same basic chemical structure as a naturally occurring amino acid. Amino acid mimetics refers to chemical compounds that have a structure that is different from the general chemical structure of an amino acid, but that function in a manner similar to a naturally occurring amino acid.

[0048] As used herein, the term "polypeptide," "peptide" and "protein," as used interchangeably herein, can be used refers to a polymer in which the monomers are amino acids and are joined together through amide bonds, alternatively referred to as a peptide. Additionally, unnatural amino acids, for example, beta-alanine, phenylglycine and homoarginine are also included. Amino acids that are not nucleic acid-encoded may also be used in the present invention. Furthermore, amino acids that have been modified to include reactive groups, glycosylation sites, polymers, therapeutic moieties, biomolecules and the like may also be used in the invention. All of the amino acids used in the present invention may be either the D- or L-isomer thereof. The L-isomer is generally preferred. In addition, other peptidomimetics are also useful in the present invention. As used herein, a "peptide" is an oligopeptide, polypeptide, peptide, protein or glycoprotein. As used herein, "polypeptide" refers to both glycosylated and unglycosylated peptides. Also included are peptides that are incompletely glycosylated by a system that expresses the peptide.

[0049] As used herein, the term "a fusion protein," also known as "a chimeric protein," refers to a protein that is translated from the joining of two or more genes that originally coded for separate proteins.

[0050] As used herein, the term "antibody", as used herein, also includes an antigen-binding fragment of a full antibody molecule. An antigen-binding fragment of an antibody may be derived, e.g., from full antibody molecules using any suitable standard techniques such as proteolytic digestion or recombinant genetic engineering techniques involving the manipulation and expression of DNA encoding antibody variable and optionally constant domains. Such DNA is known and/or is readily available from, e.g., commercial sources, DNA libraries (including, e.g., phage-antibody libraries), or can be synthesized. The DNA may be sequenced and manipulated chemically or by using molecular biology techniques, for example, to arrange one or more variable and/or constant domains into a suitable configuration, or to introduce codons, create cysteine residues, modify, add or delete amino acids, etc.

[0051] As used herein, the term "an antibody Fc region" refers to a fragment crystallizable region, which is the tail region of an antibody that interacts with cell surface receptors called

Fc receptors and some proteins of the complement system. Glycosylation of the Fc fragment is essential for Fc receptor-mediated activity.

[0052] As used herein, the term "glycoform" refers to an antibody comprising a particular glycosylation state. The term "glycosylation state" refers to an antibody having a specific or desired glycosylation pattern.

[0053] As used herein, the term "remodeling a glycan of an antibody Fc region" refers to changing or modifying the type, position, number, form, or linkage of residues in the glycan of the antibody Fc region. In one embodiment, the remodeling is lowering the number or length of core fucose.

[0054] As used herein, the term "a target glycan oxazoline" refers to a glycan oxazoline which is the glycoform to be remodeled.

[0055] **Fusion proteins**

[0056] Glycosylation pathway engineering has been developed to improve the biological function and reduce the heterogeneity of therapeutic antibodies. The way to acquire homogeneous glycoproteins is based on the strategy of glycoprotein remodeling. Fucosidases are able to catalyze the hydrolysis of an α -L fucoside. For example, the hydrolysis of the α -L fucoside involves the chemical reaction of cleaving the 1,2-linkage, 1,3-linkage, or 1,6-linkage between α -L-fucose and N-acetylglucosamine residue. On the other hand, endoglycosidases catalyze the release of oligosaccharides from glycoproteins or glycolipids. For example, the release of oligosaccharides from glycoproteins is performed by hydrolyzing the β -1,4-di-N-acetylchitobiose core of the asparagine-linked glycan or hydrolyzing the endo- β -N-acetylglucosaminide on all N-linked glycans and on biantennary and sialylated glycans of AGP (α 1-acid glycoprotein). Fucosidases and endoglycosidases in combination to treat antibody glycoforms can obtain homogeneous antibodies with a well-defined glycan at the Fc region.

[0057] The present disclosure provides a fusion protein comprising a fucosidase or a truncated fragment or a mutant thereof fuses with either N-terminal end or C-terminal end of the endoglycosidase or a truncated fragment or a mutant thereof; wherein the fusion protein exhibits both the fucosidase activity and the endoglycosidase activity.

[0058] The fucosidase or a truncated fragment of mutant thereof and the endoglycosidase or a truncated fragment or a mutant thereof are fused together directly or through a linker. In one embodiment, the fucosidase or a truncated fragment or a mutant thereof directly or indirectly links to the N-terminal of the endoglycosidase or a truncated fragment or a mutant thereof. In another embodiment, the fucosidase or a truncated fragment or a mutant thereof directly or indirectly links to the C-terminal of the endoglycosidase or a truncated fragment or a mutant thereof.

[0059] Fucosidase can be in a wild type form or a modified form compared to its wild-type form, such as a truncated fragment or a mutant thereof. Examples of the fucosidase include but are not limited to *Lactobacillus casei* α -L fucosidase C (Alfc), *Bacteroides fragilis* fucosidase (BF3242), *Bacteroides thetaiotamicron* α -L-fucosidase (BT2970), *Emticicia*
 5 *oligotrophica* α -L-fucosidase (EO0918) and *Elizabethkingia miricola* α -(1-6) fucosidase (Emfuc3), or a fragment thereof, or a mutant thereof. In some embodiments, the fucosidase is *Lactobacillus casei* α -L fucosidase C and *Elizabethkingia miricola* α -(1-6) fucosidase (Emfuc3), and a truncated fragment or a mutant thereof.

[0060] Endoglycosidase can be in a wild type form or a modified form compared to its wild-
 10 type form, such as a truncated fragment or mutant of an endoglycosidase. Examples of the endoglycosidase include but are not limited to *Streptococcus pyogenes* endoglycosidase S, *Streptococcus pyogenes* endoglycosidase S2 or a fragment or a mutant thereof. In some embodiments, the endoglycosidase is *Streptococcus pyogenes* endoglycosidase S2, or a truncated fragment or a mutant thereof.

[0061] Truncated fragments of fucosidase or endoglycosidase can also be used in the present disclosure. The truncated fragments of fucosidase or endoglycosidase have been truncated at either the N-terminal end of the peptide, the C-terminal end of the peptide or at both ends, or an internal region of the peptide. In some embodiments, the truncated fragment of fucosidase or endoglycosidase is a C-terminal or N-terminal truncated fragment thereof. In one
 20 embodiment, the truncated fragment of endoglycosidase is the IgG binding domain thereof. In some further embodiments, the truncated fragment of the endoglycosidase is an IgG binding domain of *Streptococcus pyogenes* endoglycosidase S or *Streptococcus pyogenes* endoglycosidase S2.

[0062] The fucosidase or endoglycosidase can be a mutant form thereof. In one
 25 embodiment, the endoglycosidase mutant is *Streptococcus pyogenes* endoglycosidase S having a mutation at amino acid position -D233; preferably, having a mutation at amino acid position D233Q. In some embodiments, the endoglycosidase mutant is *Streptococcus pyogenes* endoglycosidase S2 having a mutation at amino acid position: T138, D182, D184, D186, D226, or T227; preferably having a T138E, T138M, T138Q, T138R, T138 M, T138L, T138H, T138N,
 30 T138K, D182Q, D184M, D184Q, D184T, D184L, D184F, D184S, D184V, D184K, D184W, E186A, D226Q, or T227Q.

[0063] In some embodiments, the fusion protein comprises *Lactobacillus casei* α -L fucosidase C fused with *Streptococcus pyogenes* endoglycosidase S, *Streptococcus pyogenes* endoglycosidase S2, an IgG binding domain of *Streptococcus pyogenes* endoglycosidase S, an
 35 IgG binding domain of *Streptococcus pyogenes* endoglycosidase S2, *Streptococcus pyogenes* endoglycosidase S having a mutation at amino acid position D233, *Streptococcus pyogenes* endoglycosidase S2 having a mutation at amino acid position T138, *Streptococcus pyogenes*

endoglycosidase S2 having a mutation at amino acid position D182, *Streptococcus pyogenes* endoglycosidase S2 having a mutation at amino acid position D184, *Streptococcus pyogenes* endoglycosidase S2 having a mutation at amino acid position D186, *Streptococcus pyogenes* endoglycosidase S2 having a mutation at amino acid position D226, or *Streptococcus pyogenes* endoglycosidase S2 having a mutation at amino acid position T227.

[0064] In some embodiments, the fusion protein comprises *Elizabethkingia miricola* α -(1-6) fucosidase fused with *Streptococcus pyogenes* endoglycosidase S, *Streptococcus pyogenes* endoglycosidase S2, an IgG binding domain of *Streptococcus pyogenes* endoglycosidase S, an IgG binding domain of *Streptococcus pyogenes* endoglycosidase S2, *Streptococcus pyogenes* endoglycosidase S having a mutation at amino acid position D233, *Streptococcus pyogenes* endoglycosidase S2 having a mutation at amino acid position T138, *Streptococcus pyogenes* endoglycosidase S2 having a mutation at amino acid position D182, *Streptococcus pyogenes* endoglycosidase S2 having a mutation at amino acid position D184, *Streptococcus pyogenes* endoglycosidase S2 having a mutation at amino acid position D186, *Streptococcus pyogenes* endoglycosidase S2 having a mutation at amino acid position D226 or *Streptococcus pyogenes* endoglycosidase S2 having a mutation at amino acid position T227.

[0065] Examples of the amino acid sequence of the fusion protein include but are not limited to SEQ ID NOs: 2, 4, 6, 8, 10, 12, 14, 16, 18, 20, 22, 24, 26, 28, 30, 32, 34, 36, 38, 40, 42, 44, 46, 48, 50, 52, 54, 56, 58, 60, 62, 64, 66, 68, 70, 72, 74, 76, 78, 80, 82, 84, 86, 88, 90, 92, 94, 96, 98, 100, 102, 104, 106, 108, 110, 112, 114, 116, 118, 120, 122, 124, 126, 128, 130, 132, 134, 136, 138, 140, 142, 144, 146, 148, 150, 152, 154, 156, 158, 160, 162, 164, 166, 168, 170, 172, 174, 176, 178, 180, 182, 184, 186, 188, 190, 192, 194, 196, 198, 200, 202, and 204, or a substantially similar sequence thereof. Preferably, the fusion protein has the amino acid sequence selected from the group consisting of SEQ ID NOs: 34, 36, 38, 40, 42, 44, 46, 48, 50, 52, 54, 56, 58, 60, 62, 64, 66, 68, 70, 72, 74, 76, 78, 80, 82, 84, 86, 88, 90, 92, 94, 96, 98, 100, 102, 104, 106, 108, 174, 176, 178, 180, 182, 184, 186, 188, 190, 192, 194, 196, 198, 200, 202, and 204, or a substantially similar sequence thereof.

[0066] As applied to polypeptides, the term "substantial similarity" or "substantially similar" applied to polypeptides means that two peptide sequences, when optimally aligned, such as by the programs GAP or BESTFIT using default gap weights, share at least 95% sequence identity, even more preferably at least 98% or 99% sequence identity. Preferably, residue positions which are not identical differ by conservative amino acid substitutions. A "conservative amino acid substitution" is one in which an amino acid residue is substituted by another amino acid residue having a side chain (R group) with similar chemical properties (e.g., charge or hydrophobicity). In general, a conservative amino acid substitution will not substantially change the functional properties of a protein. In cases where two or more amino acid sequences differ from each other by conservative substitutions, the percent sequence identity

or degree of similarity may be adjusted upwards to correct for the conservative nature of the substitution. Means for making this adjustment are well-known to those of skill in the art. Examples of groups of amino acids that have side chains with similar chemical properties include (1) aliphatic side chains: glycine, alanine, valine, leucine and isoleucine; (2) aliphatic-hydroxyl side chains: serine and threonine; (3) amide-containing side chains: asparagine and glutamine; (4) aromatic side chains: phenylalanine, tyrosine, and tryptophan; (5) basic side chains: lysine, arginine, and histidine; (6) acidic side chains: aspartate and glutamate, and (7) sulfur-containing side chains are cysteine and methionine. Preferred conservative amino acids substitution groups are: valine-leucine-isoleucine, phenylalanine-tyrosine, lysine-arginine, alanine-valine, glutamate-aspartate, and asparagine-glutamine.

[0067] Mutations may be a substitution, deletion, or insertion of one or more codons encoding the antibody or polypeptide that results in a change in the amino acid sequence as compared with the native sequence antibody or polypeptide. Standard techniques known to those of skill in the art can be used to introduce mutations in the nucleotide sequence encoding a molecule provided herein, including, for example, site-directed mutagenesis and PCR-mediated mutagenesis which results in amino acid substitutions. Insertions or deletions may optionally be in the range of about 1 to 5 amino acids. In certain embodiments, the substitution, deletion, or insertion includes fewer than 25 amino acid substitutions, fewer than 20 amino acid substitutions, fewer than 15 amino acid substitutions, fewer than 10 amino acid substitutions, fewer than 5 amino acid substitutions, fewer than 4 amino acid substitutions, fewer than 3 amino acid substitutions, or fewer than 2 amino acid substitutions relative to the original molecule. The variation allowed may be determined by systematically making insertions, deletions, or substitutions of amino acids in the sequence and testing the resulting variants for activity exhibited by the full-length or mature native sequence.

[0068] Amino acid sequence insertions include amino- and/or carboxyl-terminal fusions ranging in length from one residue to polypeptides containing a hundred or more residues, as well as intrasequence insertions of single or multiple amino acid residues. Examples of terminal insertions include an antibody with an N-terminal methionyl residue. Other insertional variants of the antibody molecule include the fusion to the N- or C-terminus of the antibody to an enzyme (e.g., for antibody-directed enzyme prodrug therapy) or a polypeptide which increases the serum half-life of the antibody.

[0069] A conservative amino acid substitution is one in which the amino acid residue is replaced with an amino acid residue having a side chain with a similar charge. Families of amino acid residues having side chains with similar charges have been defined in the art. These families include amino acids with basic side chains (e.g., lysine, arginine, histidine), acidic side chains (e.g., aspartic acid, glutamic acid), uncharged polar side chains (e.g., glycine, asparagine, glutamine, serine, threonine, tyrosine, cysteine), nonpolar side chains (e.g., alanine, valine,

leucine, isoleucine, proline, phenylalanine, methionine, tryptophan), beta-branched side chains (e.g., threonine, valine, isoleucine) and aromatic side chains (e.g., tyrosine, phenylalanine, tryptophan, histidine). Alternatively, mutations can be introduced randomly along all or part of the coding sequence, such as by saturation mutagenesis, and the resultant mutants can be screened for biological activity to identify mutants that retain activity. Following mutagenesis, the encoded protein can be expressed and the activity of the protein can be determined.

[0070] Substantial modifications in the biological properties of the antibody are accomplished by selecting substitutions that differ significantly in their effect on maintaining (a) the structure of the polypeptide backbone in the area of the substitution, for example, as a sheet or helical conformation, (b) the charge or hydrophobicity of the molecule at the target site, or (c) the bulk of the side chain.

[0071] Non-conservative substitutions entail exchanging a member of one of these classes for another class. Such substituted residues also may be introduced into the conservative substitution sites or, into the remaining (non-conserved) sites.

[0072] The mutations can be made using methods known in the art such as oligonucleotide-mediated (site-directed) mutagenesis, alanine scanning, and PCR mutagenesis. Site-directed mutagenesis (see, e.g., Carter, 1986, Biochem J. 237:1-7; and Zoller et al., 1982, Nucl. Acids Res. 10:6487-500), cassette mutagenesis (see, e.g., Wells et al., 1985, Gene 34:315-23), or other known techniques can be performed on the cloned DNA to produce the variant DNA.

[0073] Nucleic acid molecules encoding the fusion protein of the present disclosure and the expression systems producing the fusion protein of the present disclosure

[0074] The present disclosure also provides a nucleic acid molecule expressing the fusion protein as described herein.

[0075] In some embodiments of the disclosure, the nucleic acid molecule has the nucleotide sequence selected from the group consisting of SEQ ID NOs: 1, 3, 5, 7, 9, 11, 13, 15, 17, 19, 21, 23, 25, 27, 29, 31, 33, 35, 37, 39, 41, 43, 45, 47, 49, 51, 53, 55, 57, 59, 61, 63, 65, 67, 69, 71, 73, 75, 77, 79, 81, 83, 85, 87, 89, 91, 93, 95, 97, 99, 101, 103, 105, 107, 109, 111, 113, 115, 117, 119, 121, 123, 125, 127, 129, 131, 133, 135, 137, 139, 141, 143, 145, 147, 149, 151, 153, 155, 157, 159, 161, 163, 165, 167, 169, 171, 173, 175, 177, 179, 181, 183, 185, 187, 189, 191, 193, 195, 197, 199, 201, and 203, or a substantially identical sequence thereof. In some further embodiments, the nucleic acid has the nucleotide sequence selected from the group consisting of SEQ ID NOs: 33, 35, 37, 39, 41, 43, 45, 47, 49, 51, 53, 55, 57, 59, 61, 63, 65, 67, 69, 71, 73, 75, 77, 79, 81, 83, 85, 87, 89, 91, 93, 95, 97, 99, 101, 103, 105, 107, 173, 175, 177, 179, 181, 183, 185, 187, 189, 191, 193, 195, 197, 199, 201, and 203, or a substantial identical sequence thereof.

[0076] The term "substantial identity" or "substantially identical," when referring to a nucleic acid or a fragment thereof, indicates that, when optimally aligned with appropriate nucleotide insertions or deletions with another nucleic acid (or its complementary strand), there is nucleotide sequence identity in at least 95%, and more preferably at least 96%, 97%, 98% or 99% of the nucleotide bases to the entire sequence of said reference nucleic acid sequence as measured by any well-known algorithm of sequence identity, such as FASTA, BLAST or Gap, as discussed below. A nucleic acid molecule having substantial identity to a reference nucleic acid molecule may, in certain instances, encode a polypeptide having the same or substantially similar amino acid sequence as the polypeptide encoded by the reference nucleic acid molecule.

[0077] The fusion protein can be produced using various expression systems, including prokaryotic and eukaryotic expression systems. Many such systems are widely available from commercial suppliers. In one embodiment, the fusion protein may be expressed using a vector, wherein the polynucleotide encoding said fusion protein is operably linked to a promoter sequence. In one embodiment, the promoter is a constitutive promoter. In another embodiment, the promoter is an inducible promoter.

[0078] In one embodiment, the polynucleotide or vector is contained in a virus. In another embodiment, the virus is selected from the group consisting of a retrovirus, a lentivirus, an adenovirus, and an adeno-associated virus. In one preferred embodiment of the disclosure, the polynucleotide or vector is contained in an adeno-associated virus (AAV) shuttle plasmid.

[0079] **Applications of the fusion protein of the present disclosure**

[0080] The present disclosure provides a method for remodeling a glycan of a Fc region of an antibody comprising the following steps: obtaining an antibody that has a heterogeneous glycan in the Fc region of the antibody and contacting the antibody with the fusion protein as described herein.

[0081] In one embodiment of the disclosure, the method comprises:

providing an antibody having a heterogeneous glycan in the Fc region thereof, a fusion protein of the present disclosure and a target glycan oxazoline, wherein the glycan of the antibody Fc region comprises a N-acetylglucosamine (GlcNAc) residue linking to an Asn residue of Fc region; preferably, the glycan of the antibody Fc region comprises a N-acetylglucosamine (GlcNAc) residue linking to an Asn-297 of Fc region.

contacting the antibody with the fusion protein and the target glycan oxazoline linking to the antibody Fc region;

whereby a remodeled glycan of the antibody Fc region can be obtained.

[0082] In one embodiment, the target glycan oxazoline replaces a portion of the glycan of the Fc region for linking to the antibody Fc region. It is believed, though not intended to be

restricted by any theoretical that the fusion protein according to the disclosure removes the N-linked GlcNAc in the glycan of the antibody Fc region, and then a glycosylation occurs for adding the target glycan oxazoline to the antibody Fc region. Therefore, a remodeled glycan of the antibody Fc region can be obtained.

5 [0083] Examples of the remodeled glycan include but are not limited to Sia₂(α2-6)Gal₂GlcNAc₂Man₃GlcNAc₂, Sia₂(α2-6)Gal₂GlcNAc₃Man₃GlcNAc₂, Sia₂(α2-3)Gal₂GlcNAc₂Man₃GlcNAc₂, Sia₂(α2-3)Gal₂GlcNAc₃Man₃GlcNAc₂, Sia₂(α2-3/α2-6)Gal₂GlcNAc₂Man₃GlcNAc₂, Sia₂(α2-6/α2-3)Gal₂GlcNAc₂Man₃GlcNAc₂, Sia₂(α2-3/α2-6)Gal₂GlcNAc₃Man₃GlcNAc₂, Sia₂(α2-6/α2-3)Gal₂GlcNAc₃Man₃GlcNAc₂, Sia(α2-6)Gal₂GlcNAc₂Man₃GlcNAc₂, Sia(α2-3)Gal₂GlcNAc₂Man₃GlcNAc₂, Sia(α2-6)Gal₂GlcNAc₃Man₃GlcNAc₂, Sia(α2-3)Gal₂GlcNAc₃Man₃GlcNAc₂, Sia(α2-6)GalGlcNAc₂Man₃GlcNAc₂, Sia(α2-3)GalGlcNAc₂Man₃GlcNAc₂, Sia(α2-6)GalGlcNAc₃Man₃GlcNAc₂, Sia(α2-3)GalGlcNAc₃Man₃GlcNAc₂, Gal₂GlcNAc₂Man₃GlcNAc₂, GalGlcNAcMan₃GlcNAc₂, Gal₂GlcNAc₃Man₃GlcNAc₂, GalGlcNAc₂Man₃GlcNAc₂, GalGlcNAc₃Man₃GlcNAc₂, GlcNAc₃Man₃GlcNAc₂, GlcNAc₂Man₃GlcNAc₂, GlcNAcMan₃GlcNAc₂, or Man₃GlcNAc₂.

[0084] Preferably, the method further comprises purifying the antibody with Fc region having the remodeled glycan.

[0085] Aspects of the present invention are additionally described by way of the following illustrative non-limiting examples that provide a better understanding of embodiments of the present invention and of its many advantages. The following examples are included to demonstrate preferred embodiments of the invention. It should be appreciated by those of skill in the art that the techniques disclosed in the examples which follow represent techniques used in the present invention to function well in the practice of the invention, and thus can be considered to constitute preferred modes for its practice. However, those of skill in the art should appreciate, in light of the present disclosure that many changes can be made in the specific embodiments which are disclosed and still obtain a like or similar result without departing from the spirit and scope of the invention.

EXAMPLES

30 Fusion proteins

[0086] Example 1: Production of Fusion Proteins

[0087] Fusion proteins were produced that contain full length or truncated (IgG binding motif only) EndoS or EndoS2 fused to fucosidase BF3242 (Table 1), Alfc (Table 2), BT2970 (Table 3), EO0918 (Table 4), and Emfuc3 (Table 5) as shown below in Tables 1 to 5.

35 Table 1. BF3242 Fusion proteins

Enzyme Code	Construct		N.A. SEQ ID NO	A. A. SEQ ID NO
	N-terminal	C-terminal		
A-C101	EndoS ^{IgG-BD (a)}	BF3242	1	2
A-C201	EndoS2 ^{IgG-BD (a)}		3	4
D-C101	EndoS ^{WT (b)}		5	6
D-C201	EndoS ^{D233Q}		7	8
E-C101	EndoS2 ^{WT (b)}		9	10
E-C201	EndoS2 ^{T138E}		11	12
E-C204	EndoS2 ^{T138M}		13	14
E-C401	EndoS2 ^{D184M}		15	16
A-N101	BF3242	EndoS ^{IgG-BD (a)}	17	18
A-N201		EndoS2 ^{IgG-BD (a)}	19	20
D-N101		EndoS ^{WT (b)}	21	22
D-N201		EndoS ^{D233Q}	23	24
E-N101		EndoS2 ^{WT (b)}	25	26
E-N201		EndoS2 ^{T138E}	27	28
E-N204		EndoS2 ^{T138M}	29	30
E-N401		EndoS2 ^{D184M}	31	32

^a IgG binding domain of endoS or endoS2 only

^b wild type endoS or endoS2

Table 2. AlfC Fusion proteins

Enzyme Code	Construct		N.A. SEQ ID NO	A. A. SEQ ID NO
	N-terminal	C-terminal		
A-C102	EndoS ^{IgG-BD}	AlfC	33	34
A-C202	EndoS2 ^{IgG-BD}		35	36
B-C101	EndoS ^{WT}		37	38
B-C201	EndoS ^{D233Q}		39	40
C-C101	EndoS2 ^{WT}		41	42
C-C201	EndoS2 ^{T138E}		43	44
C-C202	EndoS2 ^{T138Q}		45	46
C-C203	EndoS2 ^{T138R}		47	48
C-C204	EndoS2 ^{T138M}		49	50
C-C205	EndoS2 ^{T138L}		51	52
C-C206	EndoS2 ^{T138H}		53	54
C-C207	EndoS2 ^{T138N}		55	56
C-C208	EndoS2 ^{T138K}		57	58

C-C301	EndoS2 ^{D182Q}		59	60
C-C401	EndoS2 ^{D184M}		61	62
C-C402	EndoS2 ^{D184Q}		63	64
C-C403	EndoS2 ^{D184T}		65	66
C-C404	EndoS2 ^{D184L}		67	68
C-C405	EndoS2 ^{D184F}		69	70
C-C406	EndoS2 ^{D184S}		71	72
C-C407	EndoS2 ^{D184V}		73	74
C-C408	EndoS2 ^{D184K}		75	76
C-C409	EndoS2 ^{D184W}		77	78
C-C410	EndoS2 ^{D184M} L2 ^(a)		79	80
C-C411	EndoS2 ^{D184M} L4 ^(b)		81	82
C-C501	EndoS2 ^{E186A}		83	84
C-C601	EndoS2 ^{D226Q}		85	86
C-C701	EndoS2 ^{T227Q}		87	88
A-N102	Alfc	EndoS ^{IgG-BD}	89	90
A-N202		EndoS2 ^{IgG-BD}	91	92
B-N101		EndoS ^{WT}	93	94
B-N201		EndoS ^{D233Q}	95	96
C-N101		EndoS2 ^{WT}	97	98
C-N401		EndoS2 ^{D184M}	99	100
C-N402		EndoS2 ^{D184Q}	101	102
C-N403		EndoS2 ^{D184T}	103	104
C-N404		EndoS2 ^{D184L}	105	106
C-N405		EndoS2 ^{D184F}	107	108

^(a) L2 stands for [GGGGS]₂ (linkage of fusion protein)

^(b) L4 stands for [GGGGS]₄ (linkage of fusion protein)

Table 3. BT2970 Fusion proteins

Enzyme Code	Construct		N. A. SEQ ID NO	A.A. SEQ ID NO
	N-terminal	C-terminal		
A-C103	EndoS ^{IgG-BD}	BT2970	109	110
A-C203	EndoS2 ^{IgG-BD}		111	112
F-C101	EndoS ^{WT}		113	114
F-C201	EndoS ^{D233Q}		115	116
G-C101	EndoS2 ^{WT}		117	118

G-C201	EndoS2 ^{T138E}		119	120
G-C204	EndoS2 ^{T138M}		121	122
G-C401	EndoS2 ^{D184M}		123	124
A-N103	BT2970	EndoS ^{IgG-BD}	125	126
A-N203		EndoS2 ^{IgG-BD}	127	128
F-N101		EndoS ^{WT}	129	130
F-N201		EndoS ^{D233Q}	131	132
G-N101		EndoS2 ^{WT}	133	134
G-N201		EndoS2 ^{T138E}	135	136
G-N204		EndoS2 ^{T138M}	137	138
G-N401		EndoS2 ^{D184M}	139	140

Table 4. EO0918 Fusion proteins

Enzyme Code	Construction		N. A. SEQ ID NO	A. A. SEQ ID NO
	N-terminal	C-terminal		
A-C104	EndoS ^{IgG-BD}	EO0918	141	142
A-C204	EndoS2 ^{IgG-BD}		143	144
H-C101	EndoS ^{WT}		145	146
H-C201	EndoS ^{D233Q}		147	148
I-C101	EndoS2 ^{WT}		149	150
I-C201	EndoS2 ^{T138E}		151	152
I-C204	EndoS2 ^{T138M}		153	154
I-C401	EndoS2 ^{D184M}		155	156
A-N104	EO0918	EndoS ^{IgG-BD}	157	158
A-N204		EndoS2 ^{IgG-BD}	159	160
H-N101		EndoS ^{WT}	161	162
H-N201		EndoS ^{D233Q}	163	164
I-N101		EndoS2 ^{WT}	165	166
I-N201		EndoS2 ^{T138E}	167	168
I-N204		EndoS2 ^{T138M}	169	170
I-N401		EndoS2 ^{D184M}	171	172

Table 5. Emfuc3 Fusion proteins

Enzyme Code	Construction		N. A. SEQ ID NO	A. A. SEQ ID NO
	N-terminal	C-terminal		
A-C105	EndoS ^{IgG-BD}	Emfuc3	173	174
A-C205	EndoS2 ^{IgG-BD}		175	176

J-C101	EndoS ^{WT}		177	178
J-C201	EndoS ^{D233Q}		179	180
K-C101	EndoS2 ^{WT}		181	182
K-C201	EndoS2 ^{T138E}		183	184
K-C204	EndoS2 ^{T138M}		185	186
K-C401	EndoS2 ^{D184M}		187	188
A-N105	Emfuc3	EndoS ^{IgG-BD}	189	190
A-N205		EndoS2 ^{IgG-BD}	191	192
J-N101		EndoS ^{WT}	193	194
J-N201		EndoS ^{D233Q}	195	196
K-N101		EndoS2 ^{WT}	197	198
K-N201		EndoS2 ^{T138E}	199	200
K-N204		EndoS2 ^{T138M}	201	202
K-N401		EndoS2 ^{D184M}	203	204

Remodeling a glycan of an antibody Fc region.

[0088] Example 2: Fucosidase activity assay

[0089] A test antibody, i.e., trastuzumab (TRZ), was treated with endoS2 in Tris-HCl (pH 7.0) at 37°C for 1 hour. The treated antibody was purified by adsorbing onto a protein A affinity chromatography resin (MabSelect™), washing with Tris-HCl (pH 7.4), and eluting with citric acid buffer (pH 3.0). Most of the residual glycan in the Fc region of the resulting antibody is N-acetylglucosamine-fucose (GlcNAc-Fuc). The purified antibody was treated with fusion proteins or native fucosidases in Tris-HCl buffer (pH 7.0) at 37°C for 16 h, followed by heating at 55°C for 20 min to inactivate enzymatic activities. The reaction mixtures were filtered through a 0.22 μm membrane and the treated antibodies were purified by affinity chromatography.

[0090] The levels of residual fucose were determined by analysis of intact protein mass. The ratio of TRZ-N/N (TRZ which contains two GlcNAc in the Fc region), TRZ-N/NF (TRZ which contains one GlcNAc and one GlcNAc-Fuc in the Fc region), and TRZ-NF/NF (TRZ which contains two GlcNAc-Fuc in the Fc region) was identified by the intensities of each mass peak. The results are shown below in Table 6 and in FIG. 1.

Table 6. Percentage of fucose removed from test antibody TRZ

Enzyme			Analyses of Fucose by Intact Mass Intensity				
Enzyme Code	Construction		N/N (%)	N/NF (%)	NF/NF (%)	Fucose Level (%)	De-fucose Percentage by Enzyme (%)
	N-terminal	C-terminal					
None	No enzyme		7.16	15.29	77.55	85.2	0
BF3242	BF3242		12.34	35.99	51.67	69.67	18.22
E-C401	EndoS2 ^{D184M}	BF3242	10.78	32.5	56.72	72.97	14.35
E-N401	BF3242	EndoS2 ^{D184M}	8.72	24.29	66.99	79.14	7.11
A-N201	BF3242	EndoS2 ^{IgG-BD}	18.23	41.14	40.63	61.2	28.17
Alfc	Alfc		13.76	37.38	48.86	67.55	20.72
C-C401	EndoS2 ^{D184M}	Alfc	80.96	14.85	4.18	11.61	86.37
C-C402	EndoS2 ^{D184Q}	Alfc	75.29	19.56	5.15	14.93	82.48
C-C403	EndoS2 ^{D184T}	Alfc	79.97	16.03	4	12.02	85.89
C-C404	EndoS2 ^{D184L}	Alfc	80.23	15.55	4.22	12	85.92
C-C405	EndoS2 ^{D184F}	Alfc	75.37	19.32	5.32	14.98	82.42
C-N401	Alfc	EndoS2 ^{D184M}	26.75	44.59	28.66	50.96	40.19
C-N402	Alfc	EndoS2 ^{D184Q}	29.94	45.13	24.93	47.5	44.25
C-N403	Alfc	EndoS2 ^{D184T}	32.9	45.14	21.96	44.53	47.73
C-N404	Alfc	EndoS2 ^{D184L}	25.85	46.22	27.93	51.04	40.09
C-N405	Alfc	EndoS2 ^{D184F}	26.67	44.25	29.08	51.21	39.89

[0091] The results showed that fucosidase activity of *Bacteroides fragilis* fucosidase BF3242 was not improved by fusing it to an *S. pyogenes* endoS2 D184M mutant. Compare BF3242 (18.22% fucose removed) to E-C401 (14.35%) and E-N401 (7.11%).

- 5 [0092] Surprisingly, the fucosidase activity of Alfc was significantly increased by fusing this enzyme to either the N-terminus or the C-terminus of a D184 mutant endoS2 enzyme. For example, Alfc removed 20.72% of fucose from the test antibody, while Alfc fused to the N-terminus of the EndoS2 D184M mutant removed 40.19 % of fucose. Compare Alfc with C-N401. More unexpectedly, Alfc fused to the C-terminus of EndoS2 D184M (C-C401)
- 10 removed as much as 86.37% of fucose from the test antibody. Notably, all of the tested Alfc fusions at the C-terminus of EndoS2 D184 mutants, i.e., D184M, D184Q, D184T, D184L, and D184F, showed an unexpectedly high fucosidase activity, as compared to fusion of Alfc at the EndoS2 mutant N-terminus.

[0093] **Example 3: Fucosidase activity of additional Alfc fusion proteins**

- 15 [0094] Additional fusion proteins having Alfc fused to the C-terminus of native or site-specific mutant EndoS2 enzymes were tested for their fucosidase activity on TRZ as described above in Example 2. The results are shown below in Table 7 and in FIG. 2.

Table 7. Percentage of fucose removed from test antibody TRZ

Enzyme			Analyses of Fucose by Intact Mass Intensity				
Enzyme Code	Construction		N/N	N/NF	NF/NF	Fucose Level	De-fucose percentage by enzyme
	N-terminal	C-terminal					
None	No enzyme		7.26	15.29	77.55	85.20	0
L23C	Alfc only		20.40	43.67	35.92	57.76	32.21
C-C101	EndoS2 ^{WT}	Alfc	84.65	12.76	2.60	8.98	89.46
C-C201	EndoS2 ^{T138K}	Alfc	84.78	12.67	2.55	8.89	89.57
C-C206	EndoS2 ^{T138H}	Alfc	83.56	13.45	2.36	9.7	88.62
C-C208	EndoS2 ^{T138L}	Alfc	84.76	12.46	2.77	9	89.44
C-C205	EndoS2 ^{T138V}	Alfc	85.68	11.62	2.70	8.51	90.01
C-C204	EndoS2 ^{T138M}	Alfc	86.63	11.08	2.29	7.83	90.31
C-C207	EndoS2 ^{T138I}	Alfc	79.54	17.38	3.08	11.77	85.16
C-C202	EndoS2 ^{T138Q}	Alfc	85.30	12.11	2.59	8.65	89.85
C-C203	EndoS2 ^{T138R}	Alfc	85.75	11.71	2.55	8.41	90.13
C-C301	EndoS2 ^{G182Q}	Alfc	81.06	15.41	3.49	11.20	86.85
C-C408	EndoS2 ^{D184K}	Alfc	85.30	11.86	2.84	8.77	89.71
C-C409	EndoS2 ^{D184V}	Alfc	81.55	14.33	4.06	11.26	85.78
C-C501	EndoS2 ^{C187A}	Alfc	84.67	12.74	2.59	8.96	89.48
C-C601	EndoS2 ^{G220Q}	Alfc	85.49	11.65	2.87	8.70	89.79
C-C701	EndoS2 ^{T221Q}	Alfc	76.58	19.91	3.51	13.47	84.19

[0095] The results showed that all of the Alfc/EndoS2 fusion proteins tested removed significantly more fucose from TRZ, as compared to Alfc alone.

[0096] Additional fusion proteins of five fucosidases with truncated EndoS2 were tested for their fucosidase activity on TRZ as described above in Example 2. The results are shown below in Table 8 and in FIG. 3.

Table 8 Percentage of fucose removed from test antibody TRZ

Enzyme			Analyses of Fucose by Heavy Chain Mass Intensity		
Enzyme Code	Construction		N (%)	NF (%)	De-fucose Percentage by Enzyme (%)
	N-terminal	C-terminal			
None	No enzyme		11.78	88.22	0
Alfc	Alfc		40.45	59.55	32.5
A-C201	EndoS2 ^{IG-BD}	BF3242	23.29	76.71	13.05
A-C202	EndoS2 ^{IG-BD}	Alfc	70.52	29.48	66.58
A-C203	EndoS2 ^{IG-BD}	BT2970	11.99	88.01	0.24
A-C204	EndoS2 ^{IG-BD}	EO0918	11.6	88.4	-0.2
A-C205	EndoS2 ^{IG-BD}	Emfuc3	33.79	66.21	24.95
A-N202	Alfc	EndoS2 ^{IG-BD}	60.24	39.76	54.93
A-N203	BT2970	EndoS2 ^{IG-BD}	11	89	-0.88
A-N204	EO0918	EndoS2 ^{IG-BD}	12.8	87.2	1.16
A-N205	Emfuc3	EndoS2 ^{IG-BD}	39.91	60.09	31.89

[0097] The de-fucose capability of Alfc was improved obviously. The fusion proteins of BT2970 & EO0918 could not remove the core-fucose of antibody.

[0098] **Example 4 Additional fusion proteins having Alfc or Emfuc3 fused to the truncated EndoS2 or site-specific mutant EndoS2 enzymes**

[0066] Additional fusion proteins having Alfc or Emfuc3 fused to the truncated EndoS2 or site-specific mutant EndoS2 enzymes were tested for their fucosidase activity on TRZ as described above in Example 2. The results are shown below in Table 9 and in FIG. 4.

Table 9. Percentage of fucose removed from test antibody TRZ

Enzyme			Analyses of Fucose by Heavy Chain Mass Intensity		
Enzyme Code	Construction		N (%)	NF (%)	De-fucose Percentage by Enzyme (%)
	N-terminal	C-terminal			
None	No enzyme		12.19	87.81	0
Alfc	Alfc		39.47	60.53	31.07
A-C202	EndoS2 ^{IgG-BD}	Alfc	69.02	30.98	64.72
A-N202	Alfc	EndoS2 ^{IgG-BD}	59.46	40.54	53.83
C-C204	EndoS2 ^{T138M}	Alfc	93.03	6.97	92.06
Emfuc3	Emfuc3		32.52	67.48	23.15
A-C205	EndoS2 ^{IgG-BD}	Emfuc3	35.52	64.48	26.57
A-N205	Emfuc3	EndoS2 ^{IgG-BD}	47.38	52.62	40.08

[0099] The de-fucose capability of Alfc could be more increased when fused to C-terminal of intact EndoS2 mutations (C-C204), than truncated EndoS2 (IgG binding domain), A-C202 & A-N202. The de-fucose capability of Emfuc3 could be improved when fused to N-terminal of truncated EndoS2 (IgG binding domain), A-N205, but not when fused to C-terminal of truncated EndoS2 (IgG binding domain), A-C205.

Example 5

[0100] Additional fusion proteins having Alfc or Emfuc3 fused to the N-terminus of truncated or site-specific mutant EndoS or EndoS enzymes were tested for their fucosidase activity on TRZ as described above in Example 2. The results are shown below in Table 10 and in FIG. 5.

Table 10. Percentage of fucose removed from test antibody TRZ

Enzyme			Analyses of Fucose by Heavy Chain Mass Intensity		
Enzyme Code	Construction		N (%)	NF (%)	De-fucose Percentage by Enzyme (%)
	N-terminal	C-terminal			
None	No enzyme		10.4	89.6	0
Alfc	Alfc		30.04	66.96	25.27
B-N201	Alfc	EndoS ^{D233Q}	36.09	63.91	28.67
Emfuc3	Emfuc3		21.6	78.4	12.5
J-N201	Emfuc3	EndoS ^{D233Q}	59.37	40.63	54.65
K-N204	Emfuc3	EndoS2 ^{T138M}	46.17	53.83	39.92
K-N401	Emfuc3	EndoS2 ^{D184M}	43.85	56.15	37.33
A-N105	Emfuc3	EndoS ^{IG-BD}	46.17	53.83	39.92

[0101] The activity of Alfc was dramatic improved after fusing with EndoS2, but not improved after fusing with EndoS. In contrast, the activity of Emfuc3 was improved more obviously after fusing with EndoS rather than EndoS2.

5 [0102] **Example 6 Additional fusion proteins having EndoS or EndoS2/Emfuc3, EndoS/BF3242, Alfc, Emfuc3 or BF3242 fucosidase**

[0103] Additional fusion proteins having EndoS or EndoS2/Emfuc3, EndoS/BF3242, Alfc, Emfuc3 or BF3242 fucosidase, enzymes were tested for their fucosidase activity on TRZ as described above in Example 2. The results are shown below in Table 11 and in FIG 6.

10 Table 11. Percentage of fucose removed from test antibody TRZ

Enzyme			Analyses of Fucose by Heavy Chain Mass Intensity		
Enzyme Code	Construction		N (%)	NF (%)	De-fucose Percentage by Enzyme (%)
	N-terminal	C-terminal			
None	No enzyme		10.12	89.88	0
Alfc	Alfc		33.81	66.19	26.36
Emfuc3	Emfuc3		22.48	77.52	13.75
K-C204	EndoS2 ^{T138M}	Emfuc3	37.19	62.81	30.12
K-C401	EndoS2 ^{D184M}	Emfuc3	38.13	61.87	31.16
J-C201	EndoS ^{D233Q}	Emfuc3	25.32	74.68	16.91
BF3242	BF3242		19.72	80.28	10.68
D-C201	EndoS ^{D233Q}	BF3242	21.83	78.17	13.03

[0104] Comparing with Example 5, more significant improvement was observed when fusing Emfuc3 with N-terminal of EndoS-mutation and EndoS2-mutation than fusing to C-terminal of endoglycosidase.

15 [0105] **Example 7 Additional fusion proteins having BF3242 fused to the truncated or site-specific mutant EndoS2 or EndoS enzymes**

[0106] Additional fusion proteins having BF3242 fused to the truncated or site-specific mutant EndoS2 or EndoS enzymes were tested for their fucosidase activity on TRZ as described above in Example 2. The results are shown below in Table 12 and in FIG. 7.

Table 12. Percentage of fucose removed from test antibody TRZ

Enzyme			Analyses of Fucose by Heavy Chain Mass Intensity		
Enzyme Code	Construction		N (%)	NF (%)	De-fucose Percentage by Enzyme (%)
	N-terminal	C-terminal			
None	No enzyme		13.05	86.95	0
BF3242	BF3242		33.67	66.33	23.71
A-C201	EndoS2 ^{IgG-BD}	BF3242	23.15	76.85	11.62
D-C201	EndoS ^{D233Q}	BF3242	23.9	76.1	12.48
E-C401	EndoS2 ^{D184M}	BF3242	30.27	69.73	19.8
A-N201	BF3242	EndoS2 ^{IgG-BD}	30.43	69.57	20
D-N201	BF3242	EndoS ^{D233Q}	32.95	67.05	22.89
E-N401	BF3242	EndoS2 ^{D184M}	27.99	72.01	17.18

[0107] The de-core-fucose capability of BF3242 could not be improved by fusing with endoglycosidase.

[0108] **Example 8 Glyco-engineering of TRZ by fusion protein, C-C401**

[0109] Test antibody TRZ was treated with fusion protein C-C401, i.e., Alfc fused to the C-terminus of endoS2 D184M, in Tris-HCl (pH 7.0) at 37°C for 16 h to 20 h. After adjusting the reaction to 30°C, a sialylated complex type glycan-oxazoline, i.e., Sia₂(α2-6)Gal₂GlcNAc₂Man₃GlcNAc-oxazoline or the non-sialylated version (Gal₂GlcNAc₂Man₃GlcNAc-oxazoline) was added and the reaction incubated for 30 min to 1 h. The glyco-engineered TRZ was purified as described above in Example 2. The resulting glyco-engineered TRZ contained two Sia₂(α2-6)Gal₂GlcNAc₂Man₃GlcNAc₂ (G2S2) or two Gal₂GlcNAc₂Man₃GlcNAc₂ (G2) in the Fc region. Samples of the reactions were removed at different time intervals and analyzed by SDS-PAGE. The results are presented in FIGs. 8A and 8B.

[0110] As shown in FIG. 8A, treatment of TRZ (lane 1) for 20 h with C-C401 removed all but the N-linked GlcNAc (lane 4) to yield TRZ-N/N (GlcNAc/GlcNAc; lane 3). The amount of TRZ-N/N glycosylated after adjusting the temperature and adding Sia₂(α2-6)Gal₂GlcNAc₂Man₃GlcNAc-oxazoline was 95%, 98%, and 98% after 10 min., 20 min., and 30 min., respectively. See lanes 5, 6, and 7. Purified glyco-engineered TRZ-G2S2/G2S2 is shown in lane 8.

[0111] Turning to FIG. 8B, it shows that, after deglycosylation of TRZ for 16 h (lane 3), the amount of TRZ-N/N glycosylated after adjusting the temperature and adding

Gal₂GlcNAc₂Man₃GlcNAc-oxazoline was 98% at all time points. See lanes 4-7. Purified glyco-engineered TRZ-G2/G2 is shown in lane 8.

[0112] **Example 9 Glyco-engineering of rituximab (RTX) by fusion protein, C-C401**

[0113] Test antibody RTX was treated with fusion protein C-C401 in Tris-HCl. After
5 adjusting the temperature to 30°C, Sia₂(α2-6)Gal₂GlcNAc₂Man₃GlcNAc-oxazoline was added and the reaction incubated for 1 h. The glycan remodeled RTX was purified as described above in Example 2. The resulting RTX contained two G2S2 in the Fc region. Samples of the reactions were removed at different time intervals and analyzed by SDS-PAGE. The results are shown in FIG. 9.

10 [0114] Treatment of RTX (lane 4) for 20 h with C-C401 removed all but the N-linked GlcNAc to yield RTX-N/N (GlcNAc/GlcNAc; lane 5). The amount of RTX-N/N glycosylated after adjusting the temperature and adding Sia₂(α2-6)Gal₂GlcNAc₂Man₃GlcNAc-oxazoline was 95%, 98%, and 98% after 10 min., 20 min., and 30 min., respectively. See lanes 6, 7, and 8. Purified glyco-engineered RTX-G2S2/G2S2 is shown in lane 9.

15 [0115] **Example 10 Glyco-engineering of TRZ by fusion protein, C-C406**

[0116] Test antibody TRZ, was treated with fusion protein C-C406, i.e., Alfc fused to the C-terminus of endoS2 D184S, in Tris-HCl (pH 7.0) at 37°C for 16 h. After adjusting the temperature to 30°C, Sia₂(α2-6)Gal₂GlcNAc₂Man₃GlcNAc-oxazoline was added and the reaction incubated for 1 h. The glycan remodeled TRZ was purified as set forth in Example
20 2, *supra*. The resulting TRZ contained two G2S2 in the Fc region. Samples of the reactions were removed at different time intervals and analyzed by SDS-PAGE. The results are shown in FIG. 10.

[0117] Treatment of TRZ (lane 1) for 16 h with C-C406 removed all but the N-linked GlcNAc to form TRZ-N/N (GlcNAc/GlcNAc; lane 4). The amount of TRZ-N/N glycosylated after
25 adjusting the temperature and adding Sia₂(α2-6)Gal₂GlcNAc₂Man₃GlcNAc-oxazoline was 90%, 95%, 98%, and 98% after 30 min., 60 min., 90 min., and 120 min., respectively. See lanes 5, 6, 7, and 8. Purified glyco-engineered TRZ-G2S2/G2S2 is shown in lane 9.

[0118] **Example 11 Glyco-engineering of TRZ by fusion protein, C-C407**

[0119] Test antibody TRZ was treated with fusion protein C-C407, i.e., Alfc fused to the C-terminus of endoS2 D184V in Tris-HCl (pH 7.0) at 37°C for 12 h. Sia₂(α2-6)Gal₂GlcNAc₂Man₃GlcNAc-oxazoline was added and the reaction incubated for 1 h. The glycan remodeled TRZ was purified as described above. The resulting TRZ contained two
30 G2S2 in the Fc region. Samples of the reactions were removed at different time intervals and analyzed by SDS-PAGE. The results are shown in FIG. 11.

[0120] Treatment of TRZ (lane 1) for 12 h with C-C407 removed all but the N-linked GlcNAc to yield TRZ-N/N (GlcNAc/GlcNAc; lane 8). The amount of TRZ-N/N glycosylated after adjusting the temperature and adding Sia₂(α2-6)Gal₂GlcNAc₂Man₃GlcNAc-oxazoline was 80%, 95%, 98%, and 98% after 30 min., 60 min., 90 min., and 120 min., respectively. See lanes 4, 5, 6, and 7. Purified glyco-engineered TRZ-G2S2/G2S2 is shown in lane 9.

[0121] **Example 12 Glyco-engineering of TRZ by fusion protein, C-C204**

[0122] Test antibody TRZ was treated with fusion protein C-C204, i.e., Alfc fused to the C-terminus of endoS2 T138M in Tris-HCl (pH 7.0) at 37°C for 16 h. Sia₂(α2-6)Gal₂GlcNAc₂Man₃GlcNAc-oxazoline was added and the reaction incubated for 4 h. The glycan remodeled TRZ (50 min) was purified as described above. The resulting TRZ contained two G2S2 in the Fc region. Samples of the reactions were removed at different time intervals and analyzed by SDS-PAGE. The results are shown in FIG. 12.

[0123] Treatment of TRZ (lane 1) for 16 h with C-C204 removed all but the N-linked GlcNAc to yield TRZ-N/N (GlcNAc/GlcNAc; lane 3). The amount of TRZ-N/N glycosylated after adjusting the temperature and adding Sia₂(α2-6)Gal₂GlcNAc₂Man₃GlcNAc-oxazoline was 80%, 95%, 98%, 98%, 95% and 90% after 15 min, 30 min, 45 min, 50 min, 120 min, and 240 min, respectively. See lanes 4, 5, 6, 7, 8, and 9. Purified glyco-engineered TRZ-G2S2/G2S2 (50 min) is shown in lane 10.

[0124] While the present disclosure has been described in conjunction with the specific embodiments set forth above, many alternatives thereto and modifications and variations thereof will be apparent to those of ordinary skill in the art. All such alternatives, modifications and variations are regarded as falling within the scope of the present disclosure.

CLAIMS

What is claimed is:

1. A fusion protein comprising a fucosidase or a truncated fragment or a mutant thereof fuses with either N-terminal end or C-terminal end of an endoglycosidase or a truncated
5 fragment or a mutant thereof; wherein the fusion protein exhibits both the fucosidase activity and the endoglycosidase activity.

2. The fusion protein of claim 1, wherein the fucosidase is *Lactobacillus casei* α -L
fucosidase C (Alfc), *Bacteroides fragilis* fucosidase (BF3242), *Bacteroides thetaiotamicron* α -
10 L-fucosidase (BT2970), *Emticicia oligotrophica* α -L-fucosidase (EO0918) and *Elizabethkingia*
miricola α -(1-6) fucosidase (Emfuc3), or a fragment thereof, or a mutant thereof.

3. The fusion protein of claim 1, wherein the fucosidase is *Lactobacillus casei* α -L
fucosidase C, *Elizabethkingia miricola* α -(1-6) fucosidase (Emfuc3), or a truncated fragment or
15 a mutant thereof.

4. The fusion protein of claim 1, wherein the endoglycosidase is *Streptococcus pyogenes*
endoglycosidase S, *Streptococcus pyogenes* endoglycosidase S2 or a fragment or a mutant
thereof.

5. The fusion protein of claim 1, wherein the truncated fragment of endoglycosidase is
the IgG binding domain thereof.

6. The fusion protein of claim 1, wherein the truncated fragment of the endoglycosidase
25 is an IgG binding domain of *Streptococcus pyogenes* endoglycosidase S or *Streptococcus*
pyogenes endoglycosidase S2.

7. The fusion protein of claim 1, wherein the endoglycosidase mutant is *Streptococcus*
pyogenes endoglycosidase S having a mutation at amino acid position D233.

8. The fusion protein of claim 7, wherein the endoglycosidase mutant is *Streptococcus*
pyogenes endoglycosidase S having a mutation at amino acid position D233Q.

9. The fusion protein of claim 1, wherein the endoglycosidase mutant is *Streptococcus*

pyogenes endoglycosidase S2 having a mutation at amino acid position T138, D182, D184, D186, D226, or T227.

10. The fusion protein of claim 1, wherein the endoglycosidase mutant is *Streptococcus pyogenes* endoglycosidase S2 having a mutation at amino acid position T138E, T138M, T138Q, T138R, T138 M, T138L, T138H, T138N, T138K, D182Q, D184M, D184Q, D184T, D184L, D184F, D184S, D184V, D184K, D184W, E186A, D226Q, or T227Q.

11. The fusion protein of claim 1, which comprises *Lactobacillus casei* α -L fucosidase C fused with *Streptococcus pyogenes* endoglycosidase S, *Streptococcus pyogenes* endoglycosidase S2, an IgG binding domain of *Streptococcus pyogenes* endoglycosidase S, an IgG binding domain of *Streptococcus pyogenes* endoglycosidase S2, *Streptococcus pyogenes* endoglycosidase S having a mutation at amino acid position D233, *Streptococcus pyogenes* endoglycosidase S2 having a mutation at amino acid position T138, *Streptococcus pyogenes* endoglycosidase S2 having a mutation at amino acid position D182, *Streptococcus pyogenes* endoglycosidase S2 having a mutation at amino acid position D184, *Streptococcus pyogenes* endoglycosidase S2 having a mutation at amino acid position D186, *Streptococcus pyogenes* endoglycosidase S2 having a mutation at amino acid position D226, or *Streptococcus pyogenes* endoglycosidase S2 having a mutation at amino acid position T227.

12. The fusion protein of claim 1, which comprises *Elizabethkingia miricola* α -(1-6) fucosidase fused with *Streptococcus pyogenes* endoglycosidase S, *Streptococcus pyogenes* endoglycosidase S2, an IgG binding domain of *Streptococcus pyogenes* endoglycosidase S, an IgG binding domain of *Streptococcus pyogenes* endoglycosidase S2, *Streptococcus pyogenes* endoglycosidase S having a mutation at amino acid position D233, *Streptococcus pyogenes* endoglycosidase S2 having a mutation at amino acid position T138, *Streptococcus pyogenes* endoglycosidase S2 having a mutation at amino acid position D182, *Streptococcus pyogenes* endoglycosidase S2 having a mutation at amino acid position D184, *Streptococcus pyogenes* endoglycosidase S2 having a mutation at amino acid position D186, *Streptococcus pyogenes* endoglycosidase S2 having a mutation at amino acid position D226 or *Streptococcus pyogenes* endoglycosidase S2 having a mutation at amino acid position T227.

13. The fusion protein of claim 1, which comprises an amino acid sequence selected from SEQ ID NOs: 2, 4, 6, 8, 10, 12, 14, 16, 18, 20, 22, 24, 26, 28, 30, 32, 34, 36, 38, 40, 42, 44, 46, 48, 50, 52, 54, 56, 58, 60, 62, 64, 66, 68, 70, 72, 74, 76, 78, 80, 82, 84, 86, 88, 90, 92, 94, 96, 98, 100, 102, 104, 106, 108, 110, 112, 114, 116, 118, 120, 122, 124, 126, 128, 130, 132, 134, 136, 138, 140, 142, 144, 146, 148, 150, 152, 154, 156, 158, 160, 162, 164, 166, 168, 170, 172,

174, 176, 178, 180, 182, 184, 186, 188, 190, 192, 194, 196, 198, 200, 202, and 204, or a substantially similar sequence thereof.

14. The fusion protein of claim 1, which comprises an amino acid sequence selected from the group consisting of SEQ ID NOs: 34, 36, 38, 40, 42, 44, 46, 48, 50, 52, 54, 56, 58, 60, 62, 64, 66, 68, 70, 72, 74, 76, 78, 80, 82, 84, 86, 88, 90, 92, 94, 96, 98, 100, 102, 104, 106, 108, 174, 176, 178, 180, 182, 184, 186, 188, 190, 192, 194, 196, 198, 200, 202, and 204, or a substantially similar sequence thereof.

15. A nucleic acid molecule expressing the fusion protein of any one of claims 1 to 14.

16. The nucleic acid molecule of claim 15, comprising a nucleotide sequence selected from the group consisting of SEQ ID NOs: 1, 3, 5, 7, 9, 11, 13, 15, 17, 19, 21, 23, 25, 27, 29, 31, 33, 35, 37, 39, 41, 43, 45, 47, 49, 51, 53, 55, 57, 59, 61, 63, 65, 67, 69, 71, 73, 75, 77, 79, 81, 83, 85, 87, 89, 91, 93, 95, 97, 99, 101, 103, 105, 107, 109, 111, 113, 115, 117, 119, 121, 123, 125, 127, 129, 131, 133, 135, 137, 139, 141, 143, 145, 147, 149, 151, 153, 155, 157, 159, 161, 163, 165, 167, 169, 171, 173, 175, 177, 179, 181, 183, 185, 187, 189, 191, 193, 195, 197, 199, 201, and 203, or a substantially identical sequence thereof.

17. A vector comprising a nucleic acid molecule of claim 15 or 16.

18. A method for remodeling a glycan of an antibody Fc region comprising the following steps: obtaining an antibody that has a heterogeneous glycan in the Fc region of the antibody and contacting the antibody with the fusion protein of any one of claims 1 to 14.

19. The method of claim 18, comprising
providing an antibody having a heterogeneous glycan in the Fc region thereof, a fusion protein of any one of claims 1-14 and a target glycan oxazoline, wherein the glycan of the antibody Fc region comprises a N-acetylglucosamine (GlcNAc) residue; and
contacting the antibody with the fusion protein of any one of claims 1-14 and the target glycan oxazoline linking to the antibody Fc region;
whereby a remodeled glycan of the antibody Fc region can be obtained.

20. The method of claim 17, which further comprises purifying the antibody having the remodeled glycan in the Fc region.

21. The method of claim 18, wherein the remodeled glycan is Sia₂(α2-6)Gal₂GlcNAc₂Man₃GlcNAc₂, Sia₂(α2-6)Gal₂GlcNAc₃Man₃GlcNAc₂, Sia₂(α2-3)Gal₂GlcNAc₂Man₃GlcNAc₂, Sia₂(α2-3)Gal₂GlcNAc₃Man₃GlcNAc₂, Sia₂(α2-3/α2-6)Gal₂GlcNAc₂Man₃GlcNAc₂, Sia₂(α2-6/α2-3)Gal₂GlcNAc₂Man₃GlcNAc₂, Sia₂(α2-3/α2-6)Gal₂GlcNAc₃Man₃GlcNAc₂, Sia₂(α2-6/α2-3)Gal₂GlcNAc₃Man₃GlcNAc₂, Sia(α2-6)Gal₂GlcNAc₂Man₃GlcNAc₂, Sia(α2-3)Gal₂GlcNAc₂Man₃GlcNAc₂, Sia(α2-6)Gal₂GlcNAc₃Man₃GlcNAc₂, Sia(α2-3)Gal₂GlcNAc₃Man₃GlcNAc₂, Sia(α2-6)GalGlcNAc₂Man₃GlcNAc₂, Sia(α2-3)GalGlcNAc₂Man₃GlcNAc₂, Sia(α2-6)GalGlcNAc₃Man₃GlcNAc₂, Sia(α2-3)GalGlcNAc₃Man₃GlcNAc₂, Gal₂GlcNAc₂Man₃GlcNAc₂, GalGlcNAcMan₃GlcNAc₂, Gal₂GlcNAc₃Man₃GlcNAc₂, GalGlcNAc₂Man₃GlcNAc₂, GalGlcNAc₃Man₃GlcNAc₂, GlcNAc₃Man₃GlcNAc₂, GlcNAc₂Man₃GlcNAc₂, GlcNAcMan₃GlcNAc₂, or Man₃GlcNAc₂.

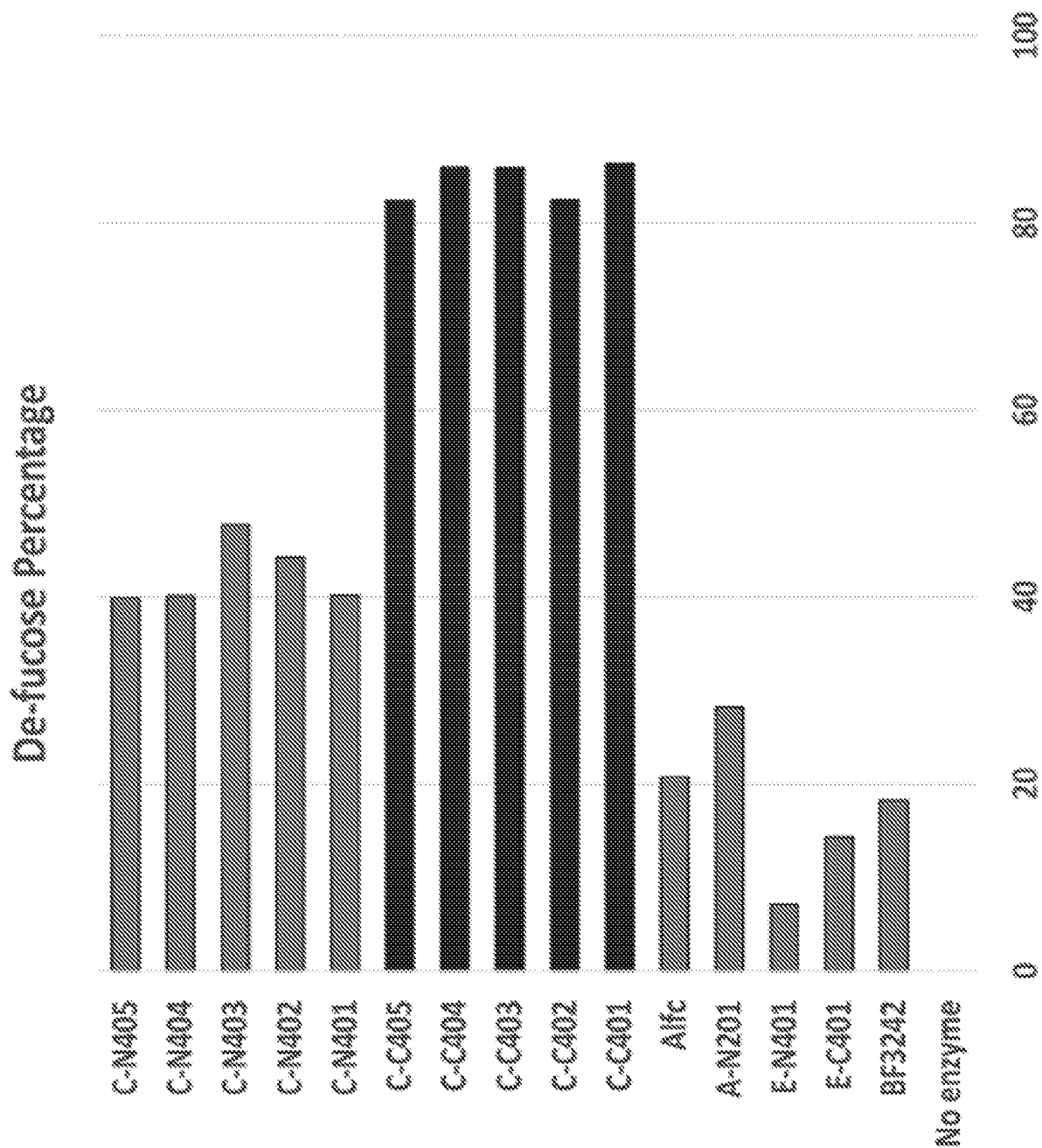


FIG. 1

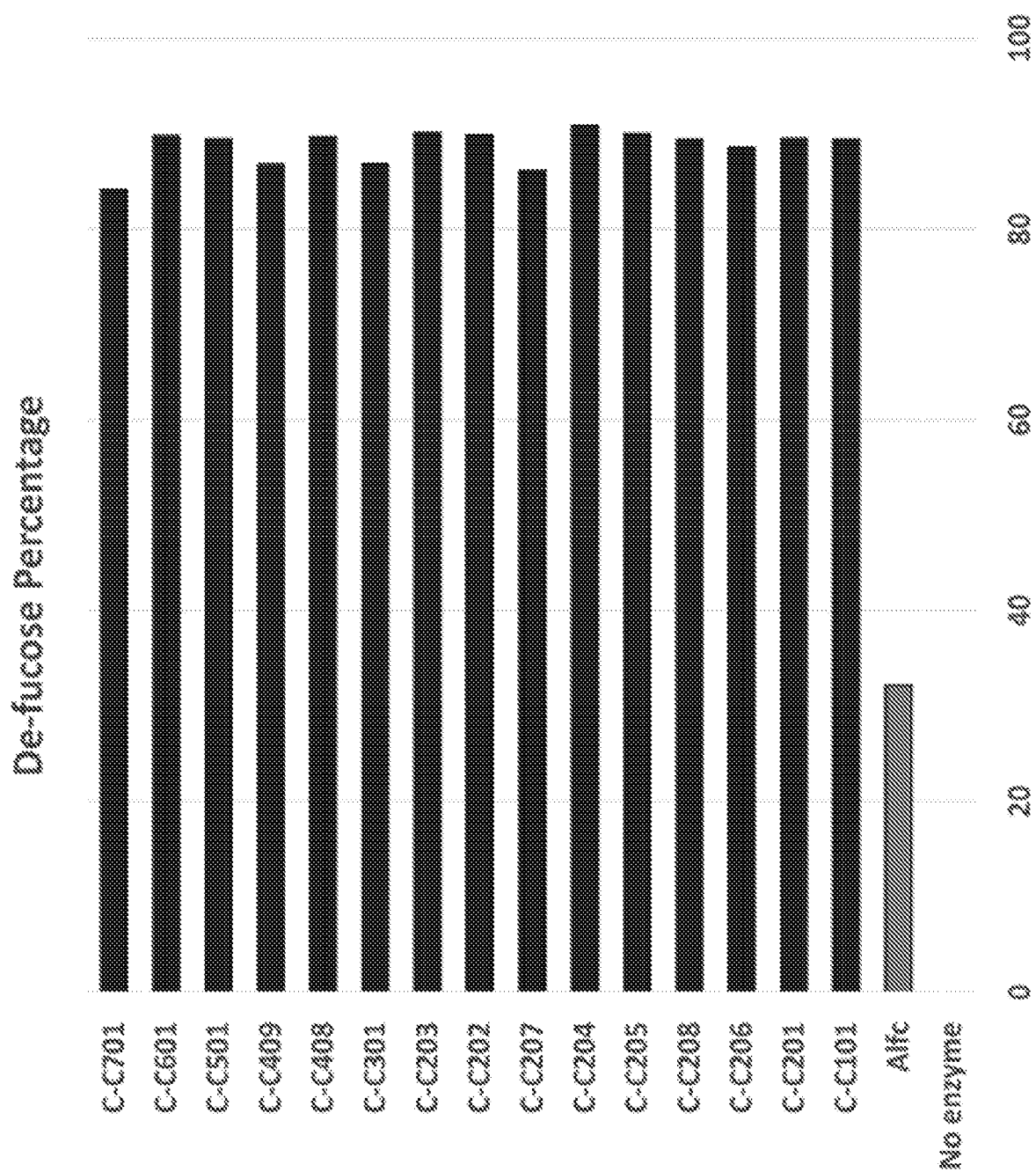


FIG. 2

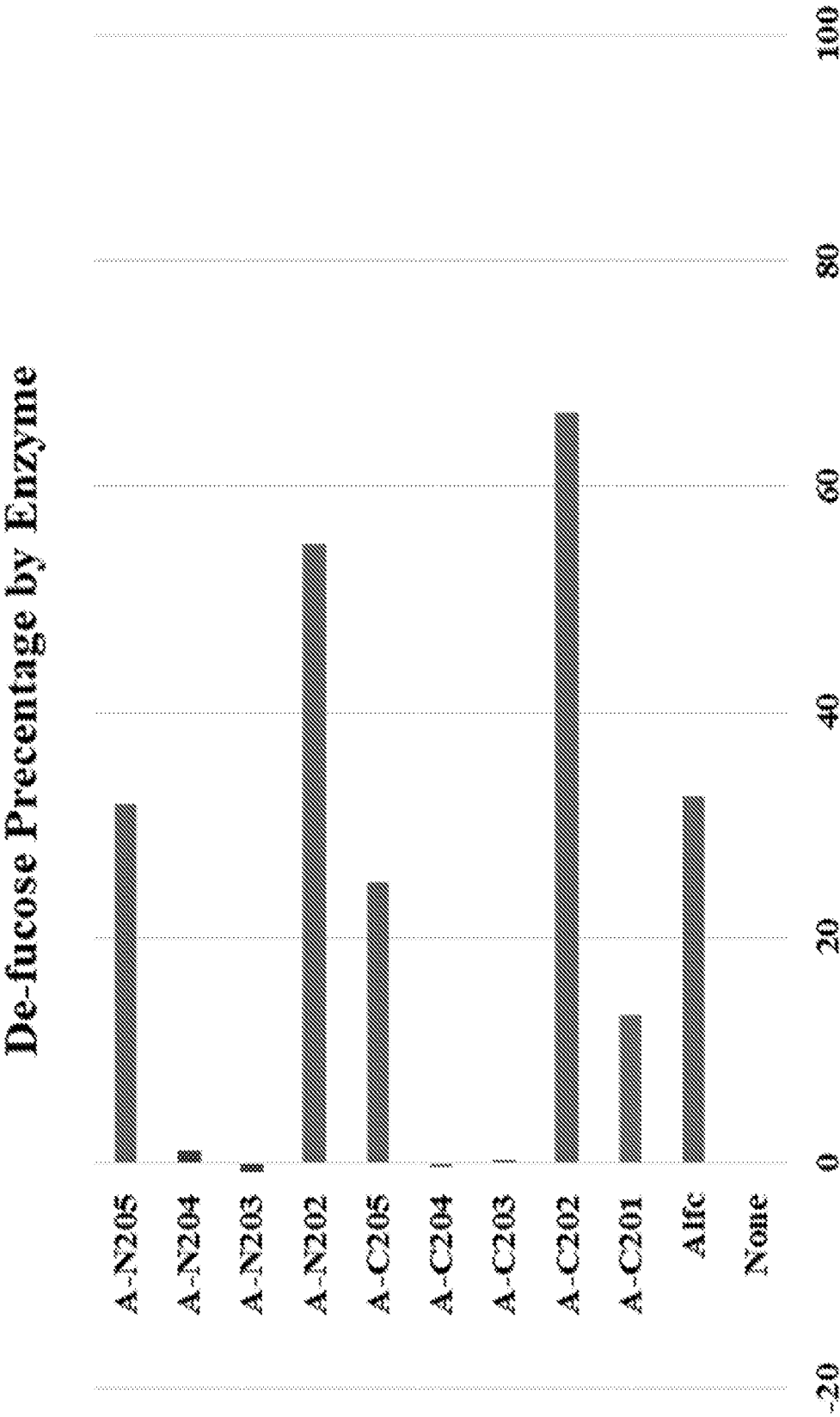


FIG. 3

De-fucose Percentage by Enzyme

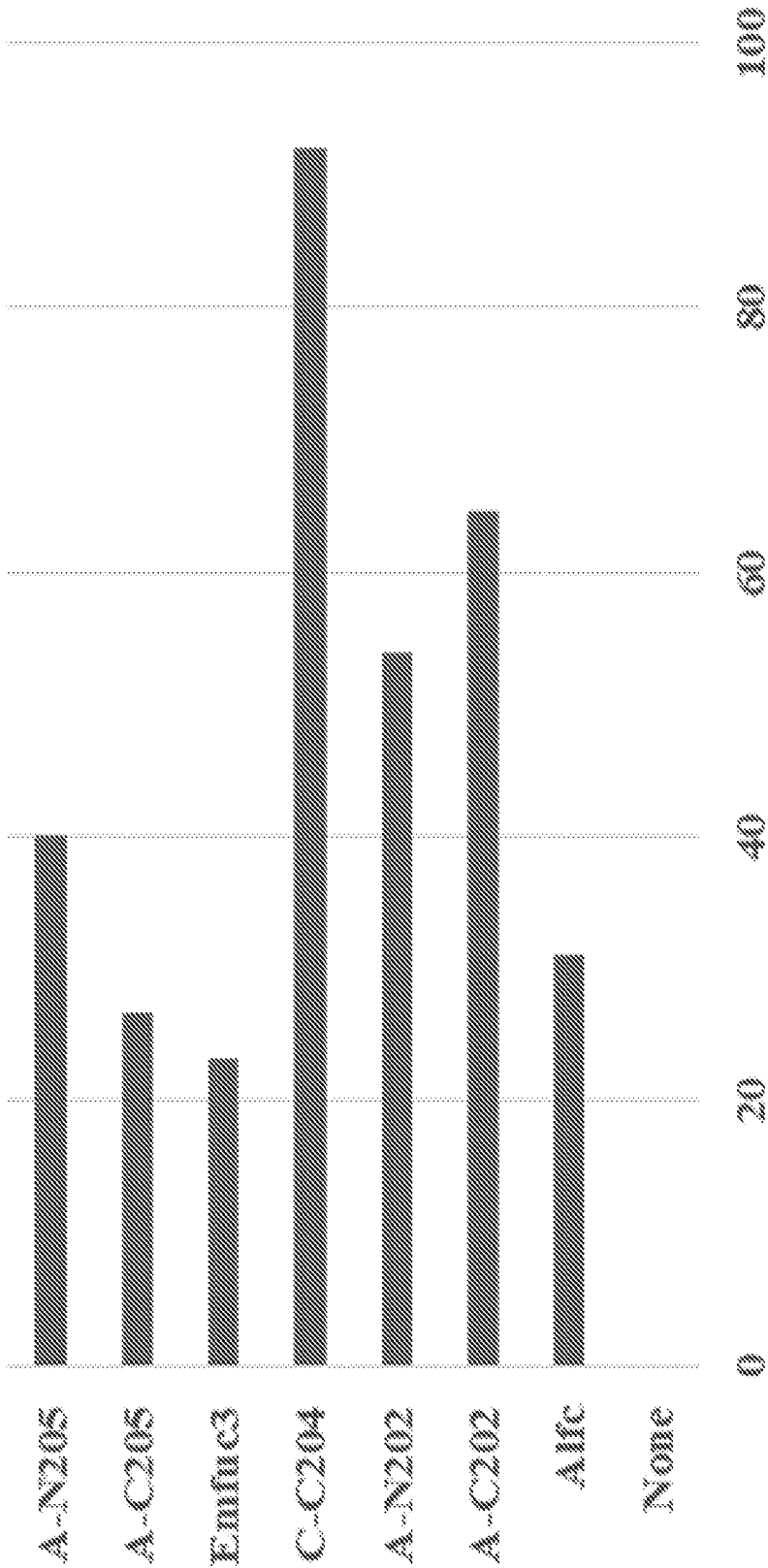


FIG. 4

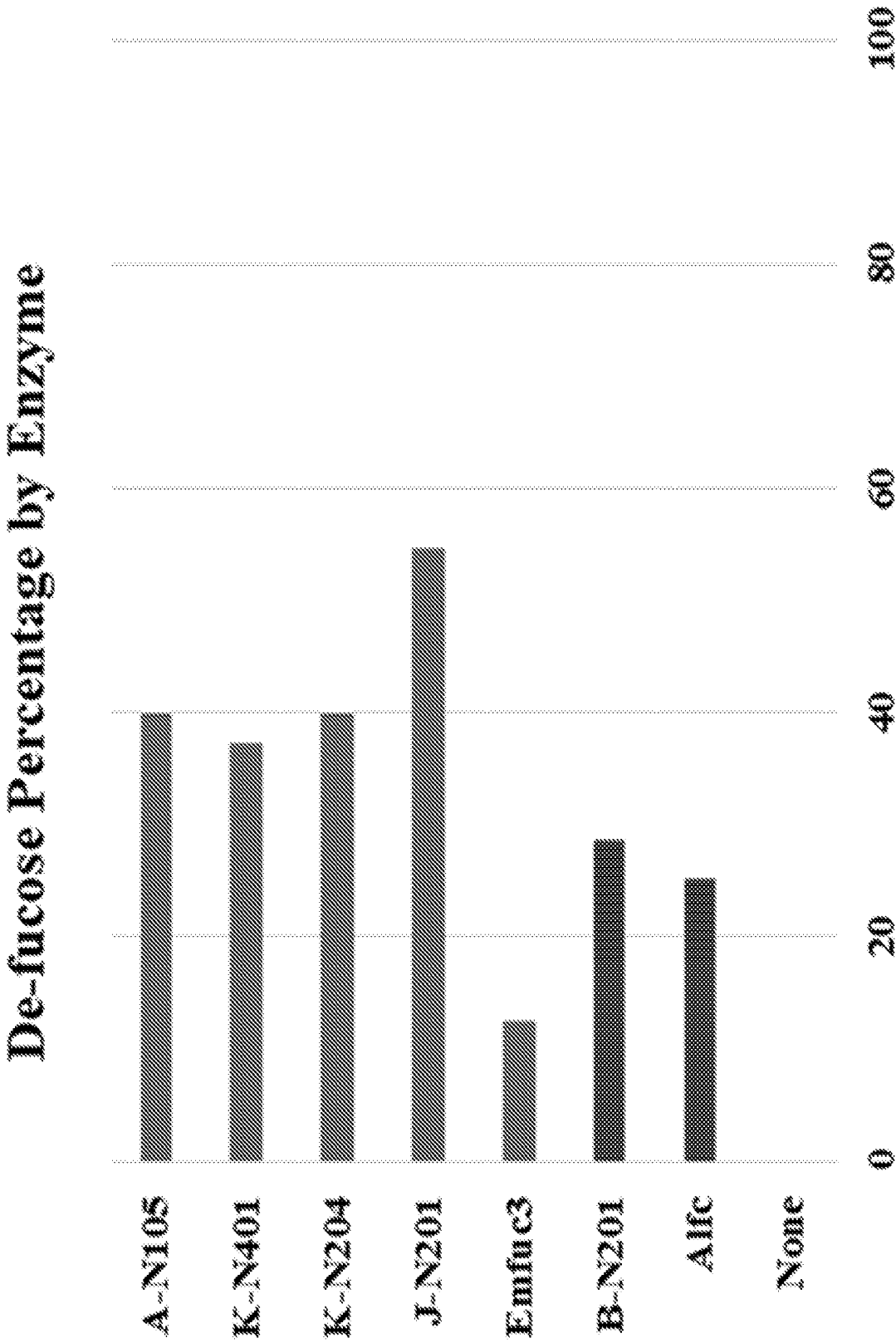
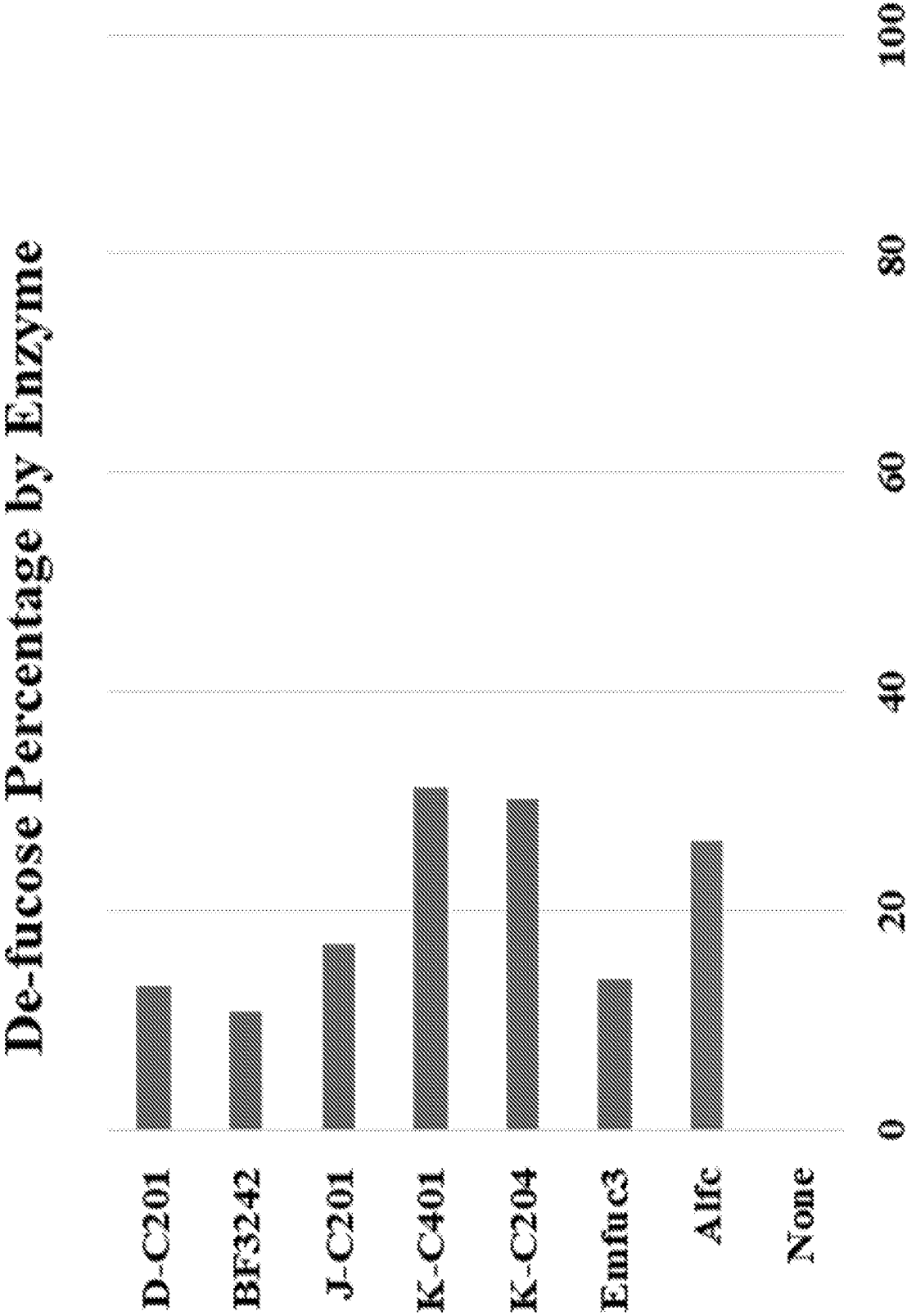


FIG. 5

FIG. 6



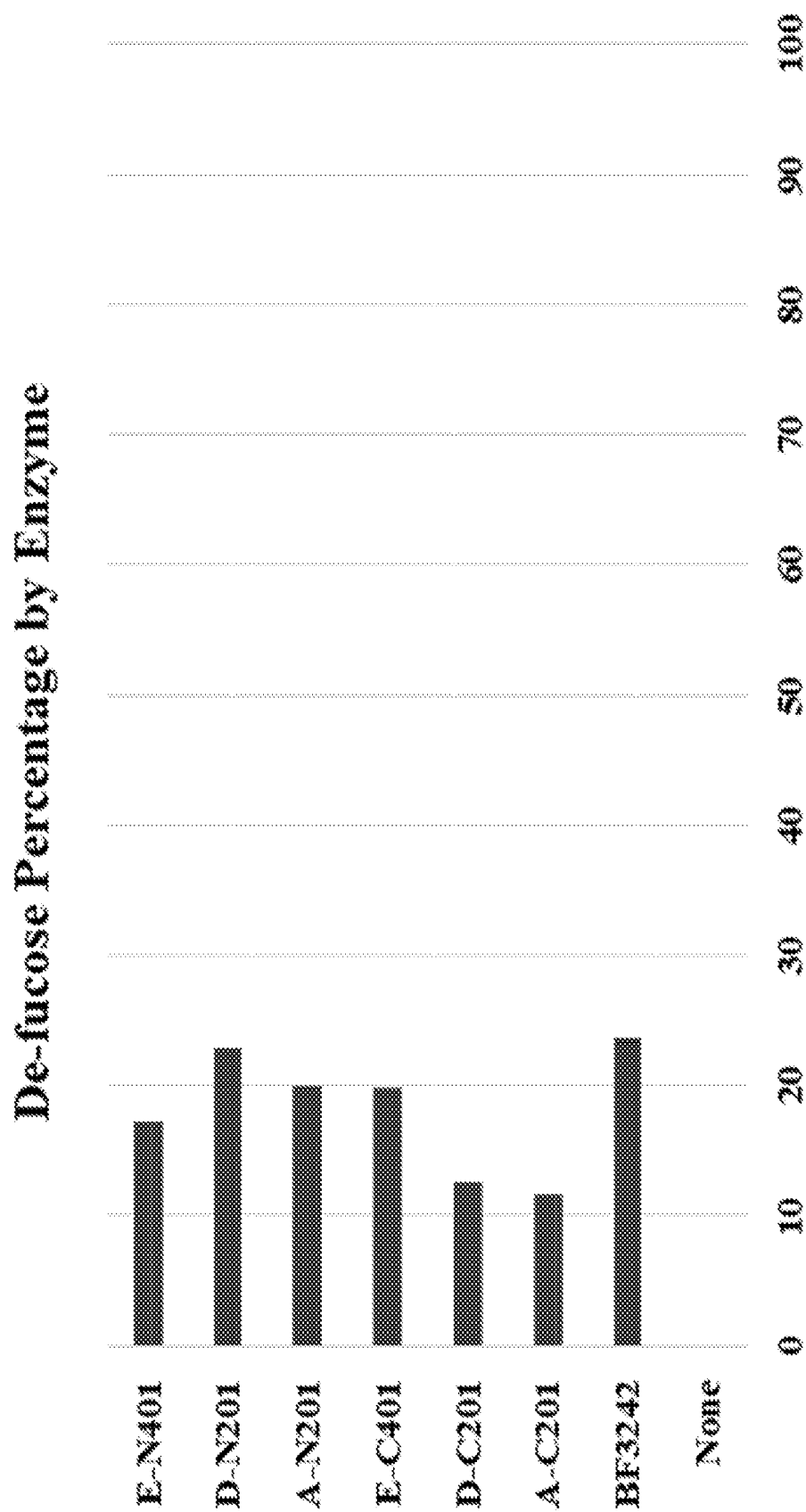


FIG. 7

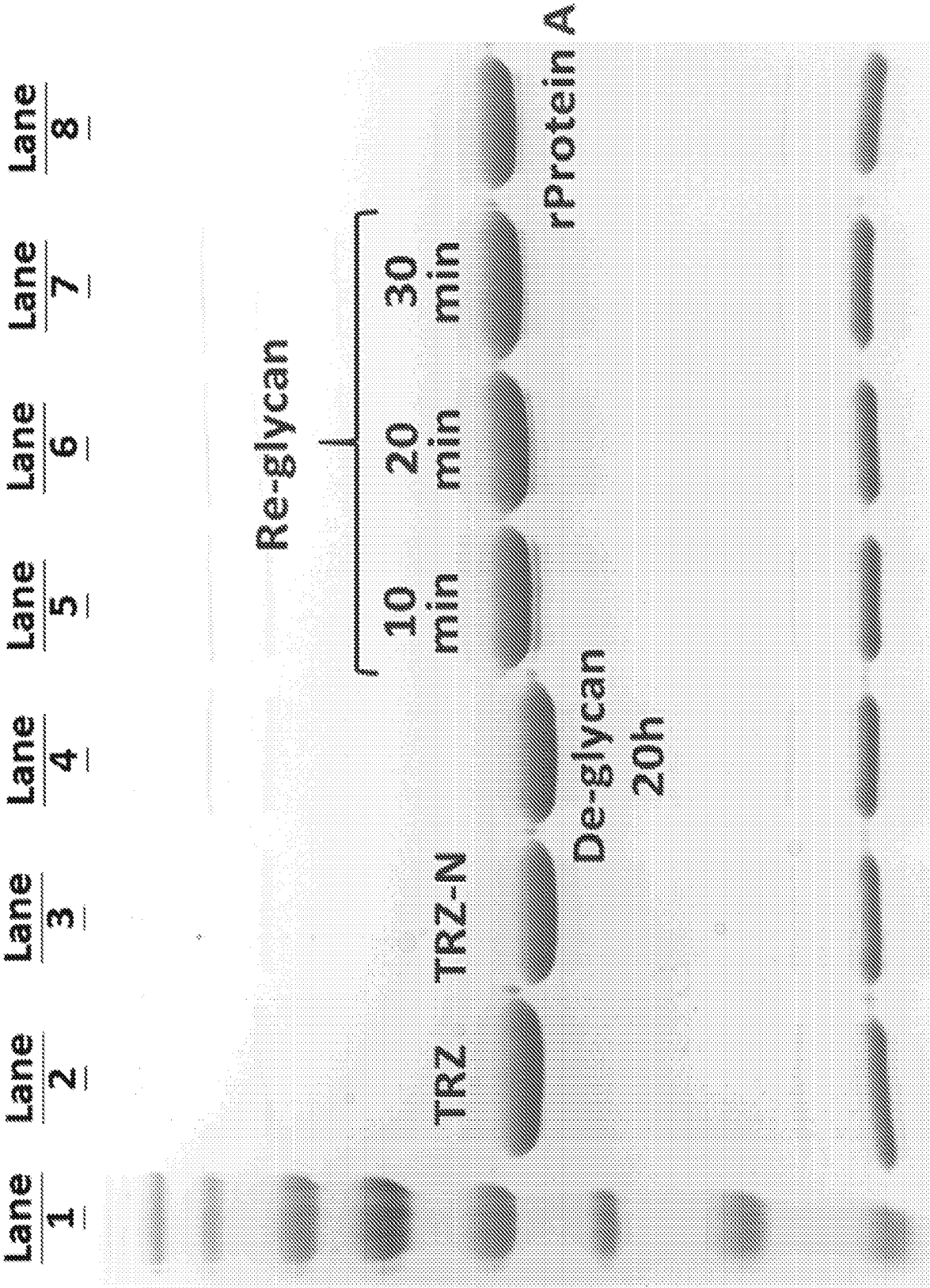


FIG. 8A

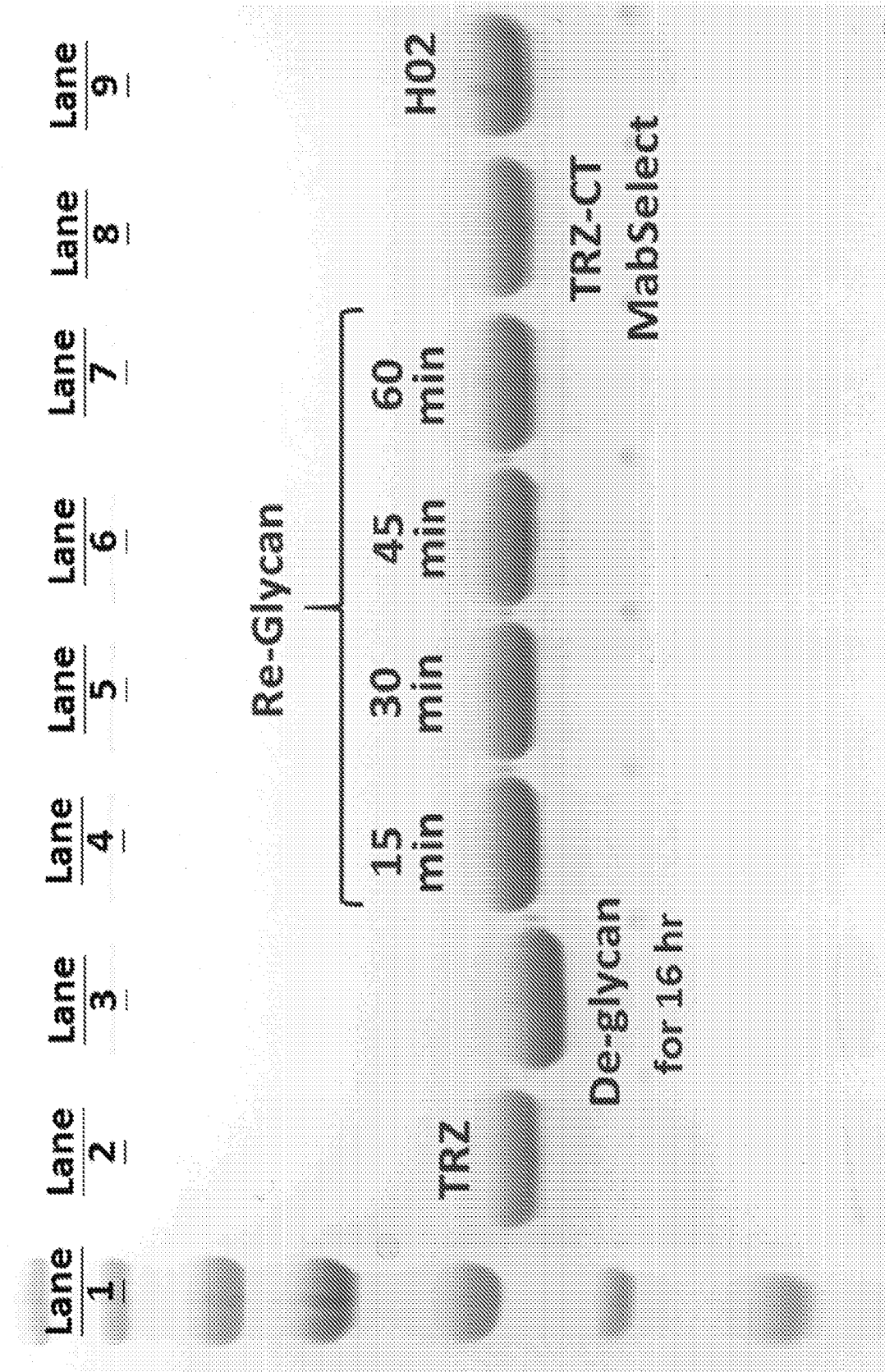


FIG. 8B

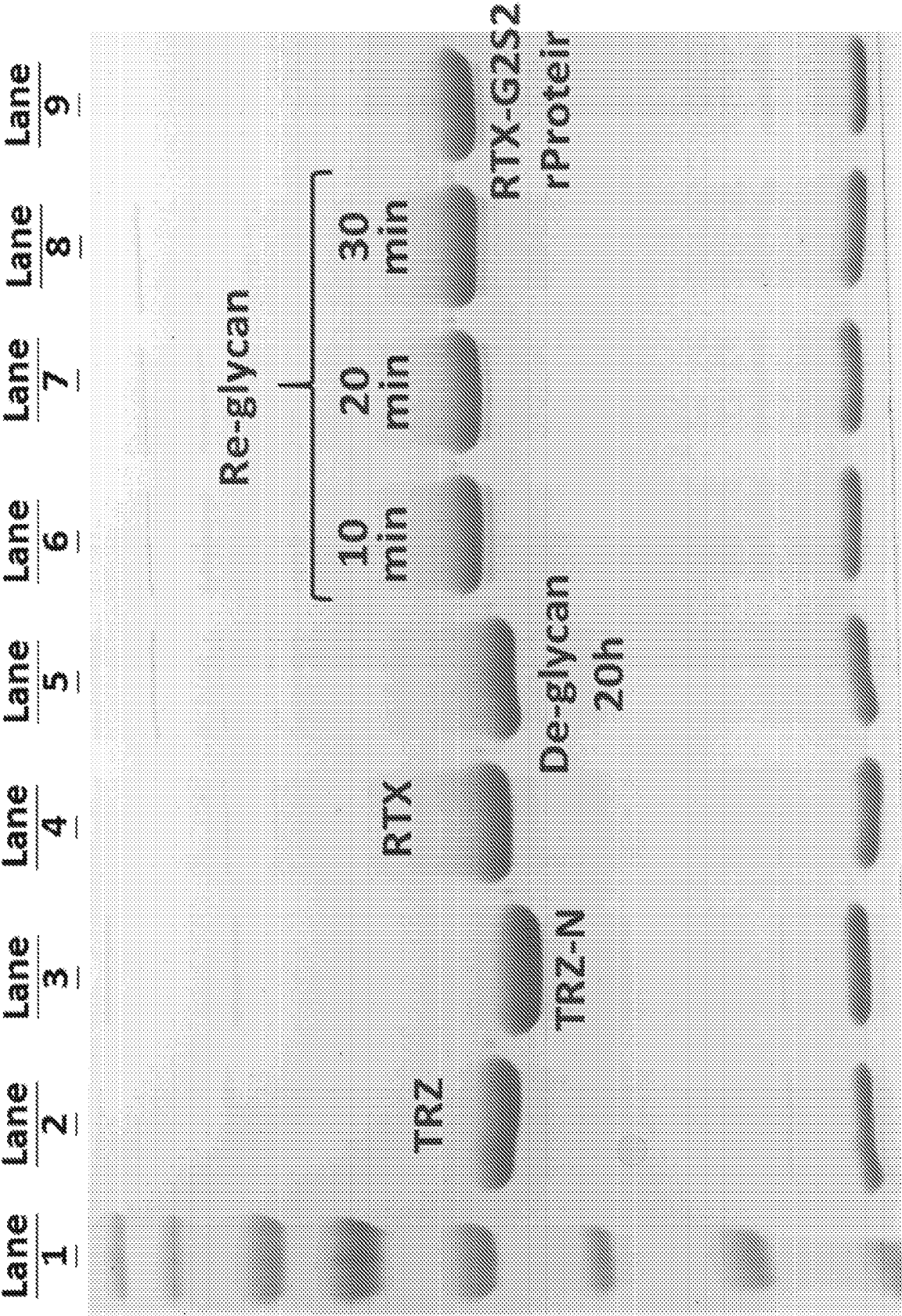


FIG. 9

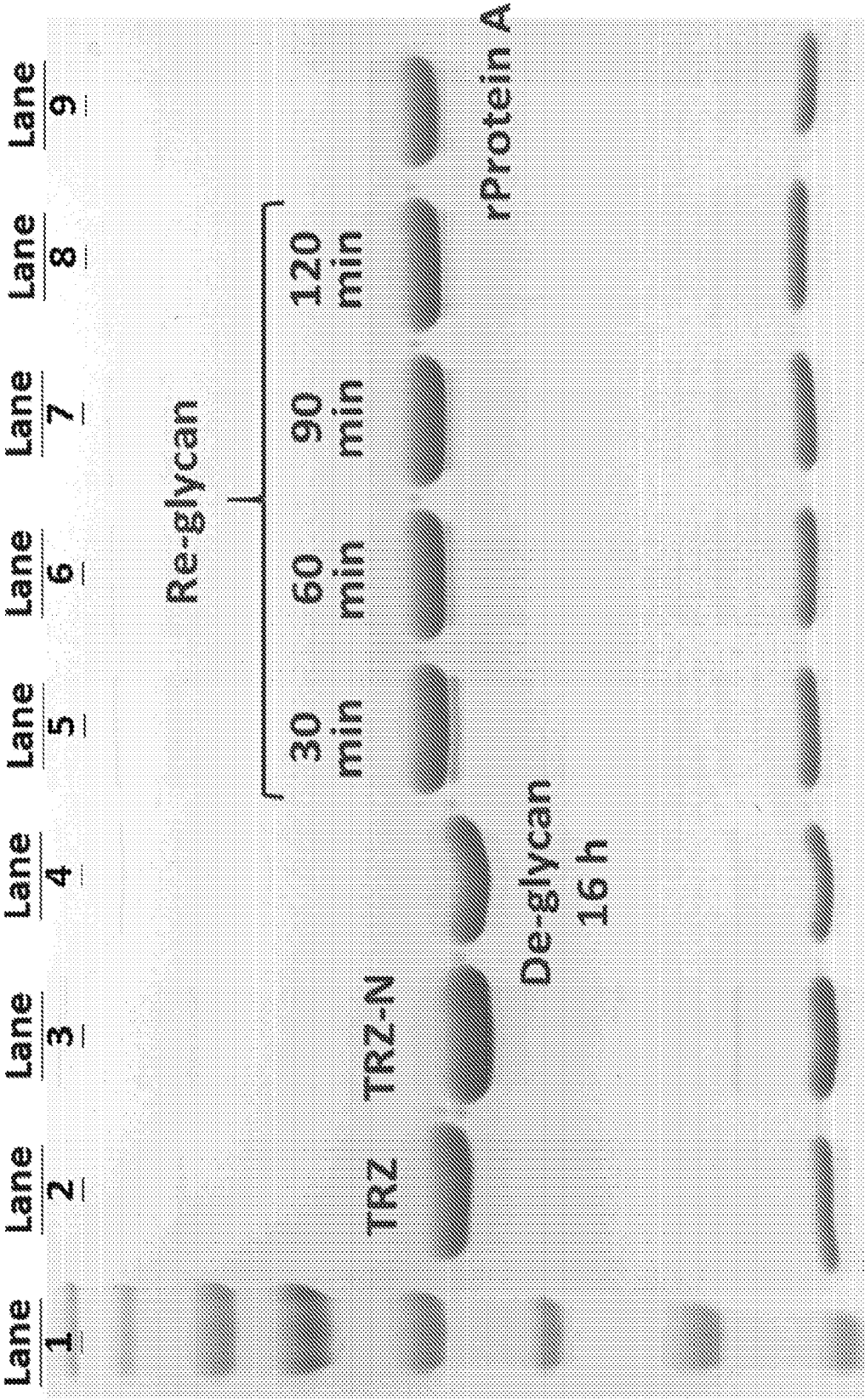


FIG. 10

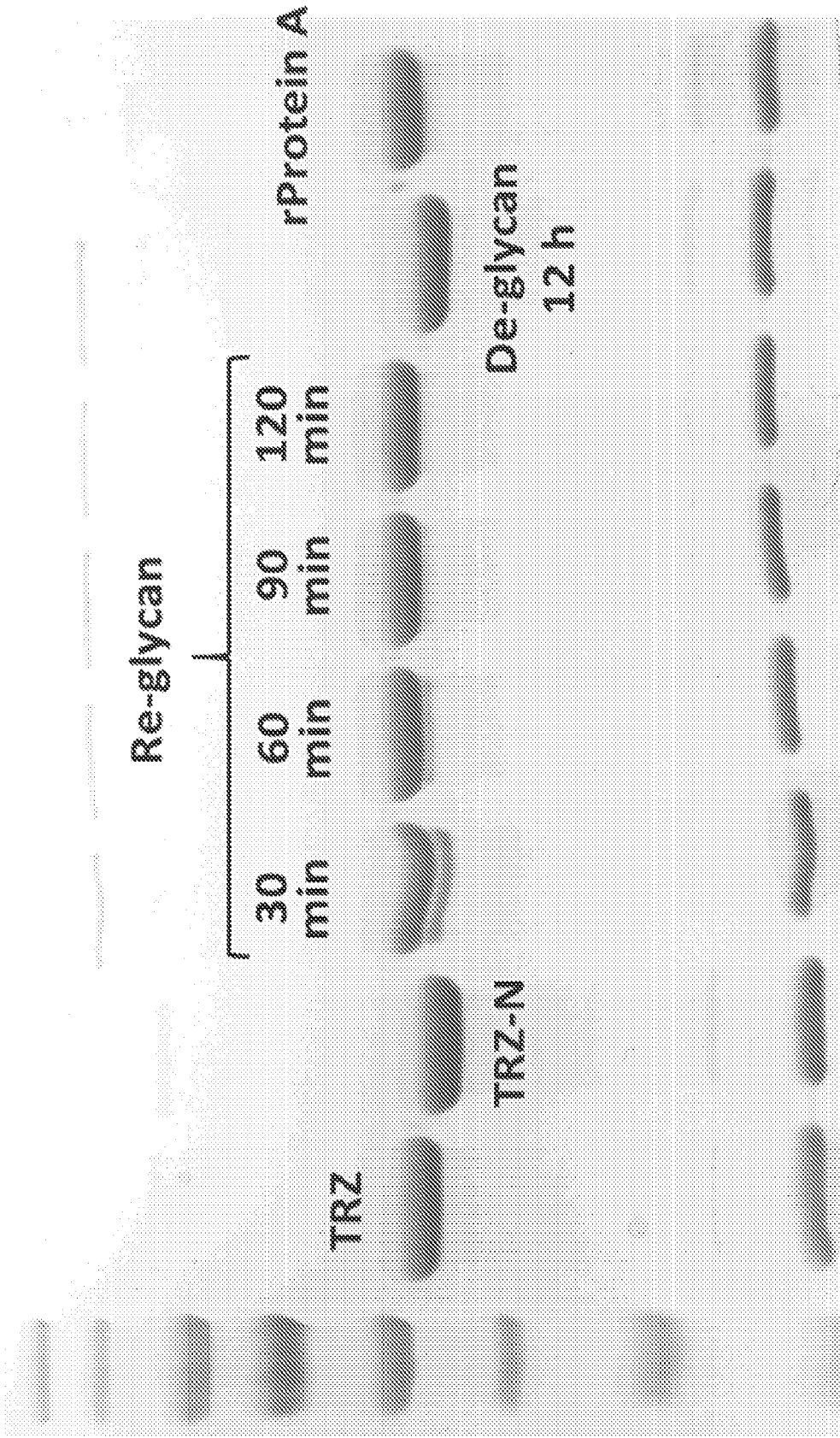


FIG. 11

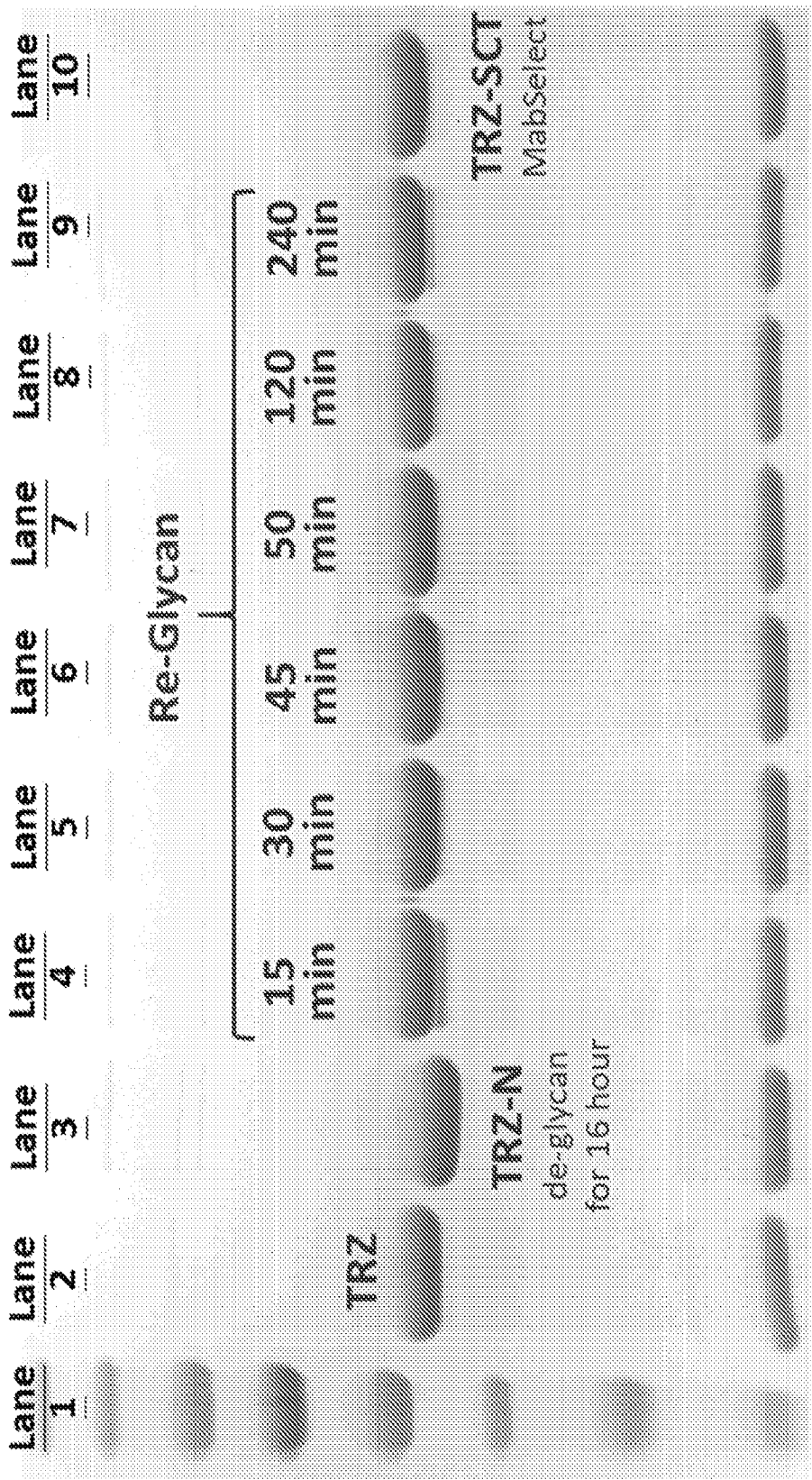


FIG. 12

A. CLASSIFICATION OF SUBJECT MATTER**C12N 9/28(2006.01)i, C12N 9/24(2006.01)i, C07K 14/195(2006.01)i, C12N 15/62(2006.01)i, C07K 16/00(2006.01)i**

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHEDMinimum documentation searched (classification system followed by classification symbols)
C12N 9/28; C12N 15/62; C12N 9/24; C12N 9/42; C12P 21/00; C07K 14/195; C07K 16/00Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched
Korean utility models and applications for utility models
Japanese utility models and applications for utility modelsElectronic data base consulted during the international search (name of data base and, where practicable, search terms used)
eKOMPASS(KIPO internal) & Keywords: fucosidase, endoglycosidase, fusion protein, hybrid, mutation, antibody glycoform, remodeling, Streptococcus pyogenes**C. DOCUMENTS CONSIDERED TO BE RELEVANT**

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	US 2019-0040374 A1 (SYNAFFIX B.V.) 07 February 2019 See abstract; paragraph [0039].	1-16, 18-21
Y	US 2018-0057804 A1 (CHO PHARMA INC. et al.) 01 March 2018 See abstract; paragraphs [0010], [0012], [0017]-[0018], [0058], [0099]-[0101], [0125].	1-16, 18-21
Y	LI, TIEZHENG et al., 'Glycosynthase mutants of endoglycosidase S2 show potent transglycosylation activity and remarkably relaxed substrate specificity for antibody glycosylation remodeling', Journal of Biological Chemistry, 2016, vol. 291, no. 32, pp. 16508-16518 See pages 16508-16511, 16514-16515.	1-16, 18-21
Y	LI, CHAO et al., 'Chemoenzymatic defucosylation of therapeutic antibodies for enhanced effector functions using bacterial α -Fucosidases', Antibody Engineering, 2018, vol. 1827, pp. 367-380 See abstract; pages 367-369.	1-16, 18-21
Y	WO 2015-184008 A1 (ACADEMIA SINICA et al.) 03 December 2015 See abstract; paragraphs [0025], [0036]-[0038], [0057]; claims 1, 5.	1-6, 15



Further documents are listed in the continuation of Box C.



See patent family annex.

* Special categories of cited documents:

"A" document defining the general state of the art which is not considered to be of particular relevance

"D" document cited by the applicant in the international application

"E" earlier application or patent but published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art

"&" document member of the same patent family

Date of the actual completion of the international search

12 November 2020 (12.11.2020)

Date of mailing of the international search report

13 November 2020 (13.11.2020)

Name and mailing address of the ISA/KR

International Application Division

Korean Intellectual Property Office

189 Cheongsa-ro, Seo-gu, Daejeon, 35208, Republic of Korea



Facsimile No. +82-42-481-8578

Authorized officer

HEO, Joo Hyung

Telephone No. +82-42-481-5373



INTERNATIONAL SEARCH REPORT

International application No.

PCT/US2020/045054**Box No. II Observations where certain claims were found unsearchable (Continuation of item 2 of first sheet)**

This international search report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. ☐ Claims Nos.:
because they relate to subject matter not required to be searched by this Authority, namely:
2. ☐ Claims Nos.:
because they relate to parts of the international application that do not comply with the prescribed requirements to such an extent that no meaningful international search can be carried out, specifically:
3. ☒ Claims Nos.: 17
because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

Box No. III Observations where unity of invention is lacking (Continuation of item 3 of first sheet)

This International Searching Authority found multiple inventions in this international application, as follows:

1. ☐ As all required additional search fees were timely paid by the applicant, this international search report covers all searchable claims.
2. ☐ As all searchable claims could be searched without effort justifying an additional fees, this Authority did not invite payment of any additional fees.
3. ☐ As only some of the required additional search fees were timely paid by the applicant, this international search report covers only those claims for which fees were paid, specifically claims Nos.:
4. ☐ No required additional search fees were timely paid by the applicant. Consequently, this international search report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.:

Remark on Protest

- ☐ The additional search fees were accompanied by the applicant's protest and, where applicable, the payment of a protest fee.
- ☐ The additional search fees were accompanied by the applicant's protest but the applicable protest fee was not paid within the time limit specified in the invitation.
- ☐ No protest accompanied the payment of additional search fees.

INTERNATIONAL SEARCH REPORT

Information on patent family members

International application No.

PCT/US2020/045054

Patent document cited in search report	Publication date	Patent family member(s)	Publication date
US 2019-0040374 A1	07/02/2019	CN 108883191 A	23/11/2018
		CN 109069658 A	21/12/2018
		EP 3413916 A1	19/12/2018
		EP 3413920 A1	19/12/2018
		EP 3413922 A1	19/12/2018
		EP 3414328 A1	19/12/2018
		JP 2017-197512 A	02/11/2017
		JP 2017-200902 A	09/11/2017
		JP 2019-507741 A	22/03/2019
		US 2019-0262467 A1	29/08/2019
		US 2019-0262468 A1	29/08/2019
		WO 2017-137423 A1	17/08/2017
		WO 2017-137456 A1	17/08/2017
		WO 2017-137457 A1	17/08/2017
US 2018-0057804 A1	01/03/2018	CN 110234341 A	13/09/2019
		EP 3532090 A1	04/09/2019
		JP 2020-500549 A	16/01/2020
		KR 10-2019-0039580 A	12/04/2019
		US 10000747 B2	19/06/2018
		US 10407673 B2	10/09/2019
		US 2018-0298361 A1	18/10/2018
		WO 2018-039373 A1	01/03/2018
WO 2015-184008 A1	03/12/2015	CN 106459920 A	22/02/2017
		CN 106573971 A	19/04/2017
		CN 107406495 A	28/11/2017
		CN 107614521 A	19/01/2018
		EP 3094352 A2	23/11/2016
		EP 3094352 B1	23/09/2020
		EP 3149036 A1	05/04/2017
		EP 3149045 A1	05/04/2017
		EP 3149161 A1	05/04/2017
		EP 3250590 A1	06/12/2017
		JP 2017-507118 A	16/03/2017
		JP 2017-517518 A	29/06/2017
		JP 2017-518989 A	13/07/2017
		JP 2020-041003 A	19/03/2020
		JP 2020-147605 A	17/09/2020
		KR 10-2016-0104727 A	05/09/2016
		KR 10-2017-0003720 A	09/01/2017
		KR 10-2017-0004023 A	10/01/2017
		KR 10-2017-0104619 A	15/09/2017
		KR 10-2017-0108146 A	26/09/2017
		US 10005847 B2	26/06/2018
		US 10118969 B2	06/11/2018
		US 10495645 B2	03/12/2019
		US 10618973 B2	14/04/2020
		US 2015-0344544 A1	03/12/2015

INTERNATIONAL SEARCH REPORT

Information on patent family members

International application No.

PCT/US2020/045054Patent document
cited in search reportPublication
datePatent family
member(s)Publication
date

US 2015-0344585 A1	03/12/2015
US 2016-0274121 A1	22/09/2016
US 2016-0280794 A1	29/09/2016
US 2019-0085062 A1	21/03/2019
US 2019-0119713 A1	25/04/2019
US 2019-0177435 A1	13/06/2019
US 2020-0078452 A1	12/03/2020
US 2020-0200758 A1	25/06/2020
US 9975965 B2	22/05/2018
US 9982041 B2	29/05/2018
WO 2015-109180 A2	23/07/2015
WO 2015-184001 A1	03/12/2015
WO 2015-184002 A1	03/12/2015
WO 2015-184004 A1	03/12/2015
WO 2015-184009 A1	03/12/2015
WO 2016-123593 A1	04/08/2016
WO 2017-100796 A1	15/06/2017