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Fragmentation stability and retention time-shift obtained by LC-MS/MS to distinguish sialylated *N*-glycan linkage isomers in therapeutic glycoproteins

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ABSTRACT

Sialylated *N*-glycan isomers with α 2-3 or α 2-6 linkage(s) have distinctive roles in glycoproteins, but are difficult to distinguish. Wild-type (WT) and glycoengineered (mutant) therapeutic glycoproteins, cytotoxic T lymphocyte-associated antigen-4-immunoglobulin (CTLA4-Ig), were produced in Chinese hamster ovary cell lines; however, their linkage isomers have not been reported. In this study, *N*-glycans of CTLA4-Igs were released, labeled with procainamide, and analyzed by liquid chromatography-tandem mass spectrometry (MS/MS) to identify and quantify sialylated *N*-glycan linkage isomers. The linkage isomers were distinguished by comparison of 1) intensity of the *N*-acetylglucosamine ion to the sialic acid ion (Ln/Nn) using different fragmentation stability in MS/MS spectra and 2) retention time-shift for a selective *m/z* value in the extracted ion chromatogram. Each isomer was distinctively identified, and each quantity (>0.1%) was obtained relative to the total *N*-glycans (100%) for all observed ionization states. Twenty sialylated *N*-glycan isomers with only α 2-3 linkage(s) in WT were identified, and each isomer's sum of quantities was 50.4%. Furthermore, 39 sialylated *N*-glycan isomers (58.8%) in mono- (3 *N*-glycans; 0.9%), bi- (18; 48.3%), tri- (14; 8.9%), and tetra- (4; 0.7%) antennary structures of mutant were obtained, which comprised mono- (15 *N*-glycans; 25.4%), di- (15; 28.4%), tri- (8; 4.8%), and tetra- (1; 0.2%) sialylation, respectively, with only α 2-3 (10 *N*-glycans; 4.8%), both α 2-3 and α 2-6 (14; 18.4%), and only α 2-6 (15; 35.6%) linkage(s). These results are consistent with those for α 2-3 neuraminidase-treated *N*-glycans. This study generated a novel plot of Ln/Nn versus retention time to distinguish sialylated *N*-glycan linkage isomers in glycoprotein.

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1. Introduction

Glycosylation is an important post-translational modification of therapeutic glycoproteins, and *N*-glycans are crucial for the physicochemical properties such as stability, solubility, bioavailability, pharmacokinetics, and immunogenicity of glycoproteins [1]. Due to its ability to generate human-like *N*-glycosylation, the Chinese hamster ovary (CHO) cell line is used to express recombinant therapeutic glycoproteins [2].

Sialic acids are mainly linked to galactose (Gal) by α 2-3 and α 2-6 linkages at the terminal position on *N*-glycans in glycoproteins. The

sialylated *N*-glycan isomers with α 2-3 and α 2-6 linkages play distinctive roles in glycoproteins with regard to biopharmaceutical efficacy [3]. *N*-acetylneuraminic acid (Neu5Ac) and *N*-glycolylneuraminic acid (Neu5Gc) are the most commonly found sialic acid derivatives in glycoproteins [4]. The hydroxyl groups of sialic acids can be substituted with *O*-acetylation, which alters the efficacy of therapeutic glycoproteins [5].

The *N*-glycans can be altered through glycoengineering, including sialylation, by controlling the conditions of cell cultures and certain glycosyltransferases [3] for the optimization of the therapeutic characteristics of glycoproteins [6], such as increased efficacy, enhanced half-life, and reduced immunogenicity [3,7]. Recombinant therapeutic glycoproteins produced in the CHO cell line only possess the α 2-3 linkage due to the absence of α 2-6 sialyltransferase (α 2-6 ST) [2], and recombinant glycoengineered immunoglobulin (Ig) G containing sialylated *N*-glycans with α 2-6

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linkage, but not α 2-3 linkage, mediates the anti-inflammatory properties [3].

Analytical tools including ultra-performance liquid chromatography (UPLC), capillary electrophoresis, matrix-assisted laser desorption/ionization (MALDI) time-of-flight mass spectrometry (MS), and liquid chromatography (LC)-electrospray ionization (ESI)-tandem MS (MS/MS) [8] can be used to obtain different structural characteristics of *N*-glycans in glycoproteins.

Of these, LC-ESI-MS/MS, given their high accuracy and reliability, have recently been used for quantitative and qualitative *N*-glycan analysis [9]. However, there are many difficulties in identifying sialylated *N*-glycans using MS: 1) the carboxyl groups of sialic acids confer negative charges to *N*-glycans, and their ionization efficiency is suppressed in the positive ion mode [10]; 2) the sialylated *N*-glycans can be attached to multiple alkali metal adducts due to their carboxyl groups, and thus complicate the interpretation of mass spectra [11]; and 3) owing to their instability, sialic acids can be lost during pretreatment and mass spectrometric steps [12]. Although analysis in a negative ion mode constitutes an approach to enhancing ionization efficiency, compared to neutral *N*-glycans, some analytical problems persist, such as loss of sialic acids, less information of fragmentation, and ionization bias of sialylated *N*-glycans [11,13]. Derivatization is used to overcome these problems, and has been reported with various methods such as glycan reducing end labeling, permethylation, and sialic acid derivatization [11].

Moreover, analytical difficulties were increased by the presence of sialyl-linkage isomers with α 2-3 and α 2-6 linkages during MS analysis [12] because of their identical mass, poor ionization, and low abundance [14,15]. The conventional method to determine sialyl-linkage isomers involves a method using linkage-specific enzyme (such as α 2-3 neuraminidase) or α 2-3- or α 2-6-linked sialic acid-binding specificity of lectin (such as *Maackia amurensis* lectin or *Sambucus nigra* lectin). However, neuraminidase digestion requires a large amount of purified samples, and lectin-binding methods usually cannot ensure sufficient binding affinity or specificity [14].

The linkage-specific derivatization of sialic acid has been utilized to form esters/carboxamides of α 2-6-linked sialic acids and lactone of α 2-3-linked sialic acids with neighboring Gal, which results in mass differences [16,17]. The recently propounded derivatization of sialic acid, including alkylamidation [12], combination of procainamide (ProA)-labeling and sialic acid derivatization via ethyl esterification and amidation [18], and methylamine and *N,N*-dimethylethylenediamine labeling [14], requires further development to address issues such as unstable lactone, harsh conditions, by-products, and loss of sialic acids and *O*-acetylation for a proper evaluation [16].

The LC-based separation using a hydrophilic interaction liquid chromatography (HILIC) column is a valuable tool because α 2-6-linked sialic acids have a stronger affinity than α 2-3-linked sialic acids [19]. Additionally, the MS/MS spectrum was used to distinguish isomers based on the relative intensity of fragment ions [20]. The different intensity of diagnostic fragment ions is attributable to the fragmentation stability of α 2-3- and α 2-6-linked sialic acids in MS/MS spectra [21,22]. Thus, the *N*-acetylglucosamine (GlcNAc) ion to the sialic acid ion ratio (Ln/Nn) was calculated as the ratio of *N*-acetylglucosamine ions to Neu5Ac ions intensities modified by the stoichiometric compensation factor which represents the number of GlcNAc and Neu5Ac for glycopeptide analysis [21].

Abatacept, which is the cytotoxic T lymphocyte-associated antigen-4 (CTLA4)-Ig, is a glycosylated fragment crystallizable (Fc)-fusion protein and is used for therapy in autoimmune diseases, such as rheumatoid arthritis [23,24]. The CTLA4 region can directly

induce T-cell downregulation and functions as an inhibitor of cluster of differentiation (CD)28-mediated T cell co-stimulation by binding CD80 and CD86 [25]. CTLA4-Ig has three *N*-glycosylation sites, namely, Asn76 and Asn108 in the CTLA4 domain, and Asn207 in the Fc region. *N*-glycans on Asn76 affect biological activities, including the binding affinity of CTLA4-Ig and protein aggregation [26]. Sialylated *N*-glycans are a major form that are attached on Asn76 and Asn108, but the Asn207 in the Fc region mainly contains complex type *N*-glycans without sialylation [24].

Some methods including overexpression of galactosyltransferase to increase Gal levels [27] and co-overexpression of α 2-3 ST or α 2-6 ST with *N*-acetylglucosaminyltransferase (GnT)-IV and GnT-V [3] in therapeutic glycoproteins have been reported to increase the antennary structures or the degree of sialylation of *N*-glycans. Recently, the glycoengineered (mutant) therapeutic glycoprotein CTLA4-Ig has been expressed for the enhancement of half-life due to an increase of contents of sialic acids in the CHO cell line with co-overexpression of GnT-IV and α 2-6 ST enzymes [7]. However, the sialylated *N*-glycan linkage isomer structures have not been reported.

Using LC-MS/MS coupled with the HILIC column, this study investigated and compared the structure and quantity of *N*-glycans, especially sialylated *N*-glycan isomers with α 2-3 and α 2-6 linkages, in mutant and wild-type (WT). The *N*-glycans were released, labeled with ProA for enhancing ionization efficiency, and compared in two different parameters generated from sialyl-linkage isomers: the ratio of intensities of fragment ions, and retention time (RT). The scatter plot of Ln/Nn value versus RT shift of *N*-glycan mass peaks based on the present results was suggested. The analysis workflow used in this study is shown in Fig. S1.

2. Materials and methods

2.1. Expression and purification of CTLA4-Igs

The WT and mutant CTLA4-Ig were expressed in CHO cells, respectively. The mutant was produced by recombinant DNA technology with the CHO cell expression system, as described previously [7]. Affinity chromatography for purification of CTLA4-Ig was performed by the ÄKTA fast protein liquid chromatography system (GE Healthcare, Piscataway, NJ, USA) with a HiTrap protein A HP (GE Healthcare) in accordance with the manufacturer's instructions.

2.2. Preparation of *N*-glycans

The *N*-glycans of WT and mutant were prepared as described previously [28]. Briefly, WT and mutant were digested by peptide-*N*-glycosidase F (New England Biolabs, Ipswich, MA, USA) at 37 °C for 24 h to release the *N*-glycans, which were then purified from proteins and salts using graphitized carbon cartridge (Alltech, Deerfield, IL, USA). The ProA (Sigma-Aldrich, St. Louis, MO, USA) was labeled with released *N*-glycans by reductive amination for fluorescent derivatization. The remaining labeling reagents were removed by solid-phase extraction using cellulose (Sigma-Aldrich). The ProA-labeled *N*-glycans were lyophilized and stored at –20 °C until further use.

2.3. Analysis of *N*-glycans using LC-MS/MS

The ProA-labeled *N*-glycan analysis was conducted by LC-ESI-high-energy collisional dissociation (HCD)-MS/MS using an Ultimate 3000 LC system (Thermo Fisher Scientific, Waltham, MA, USA) coupled with an MS (Q Exactive hybrid quadrupole orbitrap;

Thermo Fisher Scientific). 50 mM ammonium formate (pH 4.4) and 100% acetonitrile (Fisher Scientific, Hampton, NH, USA) were used as solvents A and B, respectively, for *N*-glycan separation with HILIC column (ACQUITY Glycan BEH amide column (2.1 mm × 150 mm, 1.7 μm); Waters, Milford, MA, USA). The linear gradient was set from 25% to 50% (solvent A) over 55 min at a flow rate of 0.2 mL/min, and positive ion mode ESI-MS was used. The MS parameters for analysis included full MS scan (scan range, m/z 500–3,000; resolution, 70,000; automatic gain control target, 1×10^6 ; and maximum injection time, 100 ms). The HCD-MS/MS spectra were detected from m/z 100 up to observed fragment ions, and the parameters were as follows: normalized collisional energy (NCE) 15%; resolution 17,500; automatic gain control target 2×10^5 ; and a maximum injection time 50 ms in a “Top 5” data-dependent mode. The ionization source was set with spray voltage 3.5 kV, capillary temperature 320 °C, and probe heater 300 °C. The obtained data were processed by Xcalibur 2.1 (Thermo Fisher Scientific).

2.4. Identification of *N*-glycans using LC-MS/MS

The ProA-labeled *N*-glycans were identified with elution time of the total ion chromatograms (TIC) and diagnostic fragment ions of MS/MS spectra. Data were sorted by extracted ion chromatogram (EIC) at selected m/z values, i.e., 204.0867, 366.1395, and 441.2708 for [N-acetylhexosamine (HexNAc)]⁺, [HexNAcHexose (Hex)]⁺, and [ProA-HexNAc]⁺ ions, respectively, and each MS/MS spectrum was manually interpreted by fragmentation information. Only *N*-glycans with mass errors of observed m/z within 5.0 ppm were presented, and the mass error was calculated in ppm as $[(\text{observed } m/z - \text{calculated } m/z)/\text{calculated } m/z] \times 10^6$. The calculated m/z was theoretically calculated from the molecular formula.

2.5. Identification of sialylated *N*-glycan linkage isomers

Observed *N*-glycans were confirmed to distinguish sialylated *N*-glycan isomers with α2-3 and α2-6 linkages by both RT shift of the HILIC column in EIC and the Ln/Nn value in MS/MS spectra. The EIC was used with the calculated m/z that corresponded to each identified *N*-glycan with a mass tolerance of 10.0 ppm. The peaks of sialylated *N*-glycans were observed in EIC including isomer peaks, and the intensities of oxonium ions were used to obtain Ln/Nn value in MS/MS spectra corresponding to each isomer peak. The Ln/Nn values were calculated according to a previously reported formula [21] as $([\text{GlcNAc}] \text{ ions intensity}/[\text{Neu5Ac}] \text{ ions intensity}) \times (n_{[\text{Neu5Ac}]} / n_{[\text{GlcNAc}]})$ in the MS/MS spectra. The GlcNAc ions intensity was derived from m/z 204.0867 + 366.1395, which indicated [HexNAc]⁺ ion + [HexNAcHex]⁺ ion. The Neu5Ac ions intensity was used as m/z 274.0921 + 292.1027, corresponding to [Neu5Ac–H₂O]⁺ ion + [Neu5Ac]⁺ ion. The ratio of oxonium ions intensity was divided by compensation factor as $n_{[\text{Neu5Ac}]}$ and $n_{[\text{GlcNAc}]}$, because the intensity was considered by the number of monosaccharides according to each *N*-glycan structure.

2.6. Preparation of α2-3 neuraminidase-treated *N*-glycans

To specifically release α2-3-linked sialic acids from *N*-glycans, the ProA-labeled *N*-glycans were dissolved in 50 mM sodium acetate buffer with 5 mM calcium chloride (pH 5.5) provided by the manufacturer (New England Biolabs). The α2-3 neuraminidase solution (New England Biolabs) was added with the molar ratio of 4,800:1 (substrate:enzyme), and the sample was digested at 37 °C for 1 h. The α2-3 neuraminidase-treated *N*-glycans were purified using graphitized carbon cartridge (described in Section 2.2).

2.7. Quantification of *N*-glycans

The quantity of each *N*-glycan was determined by the EIC area for all observed charge states and the adduct ions relative to the sum of the EIC area for all *N*-glycans that were generated by LC-ESI-HCD-MS/MS. The relative quantity value was expressed as the percentage (%), as reported previously [8]. The repeatability and intermediate precision of *N*-glycan analysis was performed by using relative standard deviation (RSD) according to the previous report [29]. The RSD (%) was calculated as (standard deviation/average of relative quantities) × 100.

3. Results and discussion

3.1. Structural analysis of *N*-glycans by MS/MS fragmentation

N-glycans of WT and mutant were released and labeled with ProA, and were identified by LC-ESI-HCD-MS/MS, respectively. The ESI was used for a mild ionization source and the *N*-glycan structures were determined by accurate mass and MS/MS spectra with fragment ions generated in HCD [5]. The MS/MS spectrum for identification of *N*-glycan structure and distinguishing of sialylated *N*-glycan isomers with α2-3 and α2-6 linkages are presented in Fig. 1.

Fig. 1A shows the MS/MS spectrum of *N*-glycans (no. 20, Table S1) as [ProA-HexNAc₄Hex₅Fucose (Fuc)₁Neu5Ac₂] from mutant. The oxonium ions which indicate abundant and diagnostic fragment ions derived from *N*-glycan were observed with [HexNAc]⁺ (observed m/z = 204.0873), [HexNAcHex]⁺ (observed m/z = 366.1409), and [ProA-HexNAc]⁺ (observed m/z = 441.2723). The MS/MS spectrum in Fig. 1A provides other oxonium ions at m/z = 274.0927 and 292.1032, which correspond to the [Neu5Ac–H₂O]⁺ and [Neu5Ac]⁺ ions, respectively, and indicate the presence of Neu5Ac. The fragment ion [ProA-HexNAcFuc]⁺ (observed m/z = 587.3301) was detected and showed the mass difference of 146.0578 from [ProA-HexNAc]⁺ which corresponded to the Fuc, and this *N*-glycan was determined through core-fucosylation and sialylation.

Other fragment ions were characterized by sequential mass difference, such as HexNAc, Hex, and Fuc from the [ProA-HexNAc]⁺ ion. The [ProA-HexNAc₂Fuc₁]⁺ ion (observed m/z = 790.4089), [ProA-HexNAc₂Hex₁Fuc₁]⁺ ion (observed m/z = 952.4609), and [ProA-HexNAc₂Hex₂Fuc₁]⁺ ion (observed m/z = 1114.5155) were consecutively observed up to the largest fragment ion [ProA-HexNAc₃Hex₄Fuc₁Neu5Ac₁]⁺ (observed m/z = 1932.8356). The mass difference between the precursor ion (observed m/z = 1295.0169, with doubly charged states in full MS scan, data not shown) and the largest fragment ion [ProA-HexNAc₃Hex₄Fuc₁Neu5Ac₁]⁺ was identified with presence of [HexNAcHexNeu5Ac], which determined the structure of the precursor ion as the [ProA-HexNAc₄Hex₅Fuc₁Neu5Ac₂]⁺ ion. These results indicated that the *N*-glycan of Fig. 1A is a bi-antennary disialylated *N*-glycan. All mass errors of precursor and oxonium ions were <5.0 ppm, which indicated high accuracy.

The remaining *N*-glycans were identified using similar methods. Briefly, the oxonium ions of *N*-glycans for [HexNAc]⁺, [HexNAcHex]⁺, and [ProA-HexNAc]⁺ ions were detected in the MS/MS spectra, and the characteristic oxonium ions for [Neu5Ac]⁺ (Fig. 1), [Neu5Ac–OAc₂]⁺ (di-O-acetylated Neu5Ac ion, calculated m/z = 376.1238; Fig. S2A), [Neu5Ac–OAc]⁺ (mono-O-acetylated Neu5Ac ion, calculated m/z = 334.1133; Fig. S2B), or [Neu5Gc]⁺ ions (calculated m/z = 308.0976) were diagnostically used to identify sialic acid derivatives. The core-fucosylation ion migrated to the terminal region during MS/MS fragmentation; however, the [ProA-HexNAcFuc]⁺ ion was detected without migration of Fuc as ProA-labeling inhibited this migration [30].

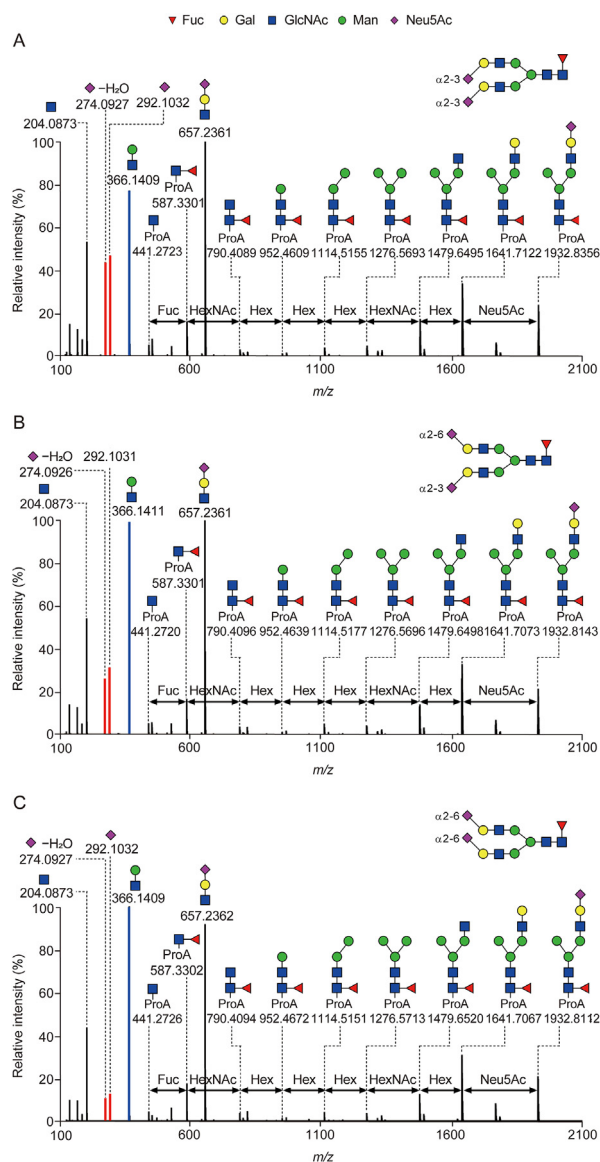


Fig. 1. Tandem mass spectrometry (MS/MS) spectra of procainamide (ProA)-labeled *N*-glycans. The bi-antennary di-sialylated *N*-glycans in mutant with (A) α 2-3/ α 2-3 linkages (no. 20), (B) α 2-3/ α 2-6 linkages (no. 20a), and (C) α 2-6/ α 2-6 linkages (no. 20b) were identified. The oxonium ions are indicated by the two red lines for *N*-acetylneuraminic acid ions (Neu5Ac), whereas the blue line represents [HexNAcHex]⁺ ion. Fuc: fucose; Gal: galactose; GlcNAc: *N*-acetylglucosamine; Man: mannose; Neu5Ac: *N*-acetylneuraminic acid; HexNAc: *N*-acetylhexosamine; Hex: hexose.

Glycan reducing end labeling is a standard method for attaching fluorescent reagents to *N*-glycans by reductive amination, and the 2-aminobenzamide (AB) is well-established and the most frequently used [11,31]. However, the AB-labeling showed poor ionization efficiency in ESI-MS, while ionization efficiency of ProA-labeling improved to 10–50 times higher than that of AB-labeling due to high proton affinity of tertiary amine moiety [31,32]. Additionally, MS/MS spectra provided larger fragment ions in ProA-labeled *N*-glycans than AB-labeled *N*-glycans, and those can enable accurate interpretation of *N*-glycan structures [31].

3.2. Identification of sialylated *N*-glycan linkage isomers by fragmentation stability in MS/MS spectra

The MS/MS spectra in Figs. 1B and C showed identical *m/z* values with fragment and precursor ions (observed *m/z* = 1295.0156 and

1295.0169 in full MS scan, data not shown) that are shown in Fig. 1A. The oxonium ions for [HexNAc]⁺, [HexNAcHex]⁺, [ProA-HexNAc]⁺, [Neu5Ac]⁺, and [Neu5Ac–H₂O]⁺ were detected as sialylated *N*-glycans, and the structure of precursor ion was identified with the [ProA-HexNAc₄Hex₅Fuc₁Neu5Ac₂]⁺ ion using sequentially fragmented ions (Figs. 1B and C). Thus, the *N*-glycans were determined as comprising bi-antennary di-sialylated *N*-glycans by confirming MS/MS fragments of the same *N*-glycan composition as shown in Fig. 1A. However, the relative intensities (%) of [Neu5Ac–H₂O]⁺ and [Neu5Ac]⁺ ions (calculated *m/z* = 274.0921 and 292.1027, two red lines in Fig. 1) related to sialic acids were obtained as 43.7% and 47.0% (Fig. 1A), 26.2% and 31.5% (Fig. 1B), and 10.4% and 12.7% (Fig. 1C), respectively. Additionally, the relative intensities of [HexNAcHex]⁺ ion (calculated *m/z* = 366.1395, blue line in Fig. 1) were obtained with the different intensities, as 77.2% (Fig. 1A), 99.3% (Fig. 1B), and 100.0% (Fig. 1C), respectively. Although the MS/MS spectrum showed generally poor specificity for distinguishing among isomers, the relative intensities of diagnostic fragment ions, such as sialic acid ions and HexNAcHex ion, were used to identify isomers, as a result of different fragmentation routes and branching ratios [20]. The intensity of MS peak is mainly dependent on the efficiency of ionization and cleavage that generates the fragment ion of each *N*-glycan [33]. The different relative intensities in sialic acids and HexNAcHex ion showed the isomers related to sialic acids. Additionally, the tendency of oxonium ions was observed in accordance with a previous report owing to the sialyl-linkage isomers of *N*-glycopeptide in the MS/MS spectrum, and indicated relative intensities of lower Neu5Ac ions and higher HexNAcHex ion in α 2-6 linkage(s) than those in the α 2-3 linkage(s) [21].

The differences of the three MS/MS spectra in Fig. 1 indicate that *N*-glycans identified with di-sialylation had different sialyl-linkage isomers with the possibility of α 2-3/ α 2-3 (Fig. 1A), α 2-3/ α 2-6 (Fig. 1B), and α 2-6/ α 2-6 linkages (Fig. 1C). The relative intensities of *N*-glycans related to sialic acids decreased, whereas core and antennary (HexNAcHex) structures were increased in the order of α 2-3/ α 2-3, α 2-3/ α 2-6, and α 2-6/ α 2-6 linkages in the same composition of *N*-glycans. Furthermore, the relative intensities (%) of [Neu5Ac–H₂O]⁺, [Neu5Ac]⁺, and [HexNAcHex]⁺ ions of no. 20 from WT (as only α 2-3 linkages) and no. 20b from the α 2-3 neuraminidase-treated mutant (as only α 2-6 linkages) were compared to investigate the usefulness of these results. The MS/MS spectrum of WT (Fig. S3A) showed similar relative intensities to those of Fig. 1A, in sialic acid (45.3% and 49.9%; red lines) and [HexNAcHex]⁺ (77.0%; blue line) ions as well as the same *N*-glycan composition and similar fragment ions. The *N*-glycan as no. 20b from the α 2-3 neuraminidase-treated mutant was detected with relative intensities of sialic acids (10.4% and 12.4%; red lines) and [HexNAcHex]⁺ (100.0%; blue line) ions in the MS/MS spectrum (Fig. S3B), which was similar to those in Fig. 1C. These results indicated that the di-sialylated *N*-glycans in mutant were identified as α 2-3/ α 2-3 (Fig. 1A), α 2-3/ α 2-6 (Fig. 1B), and α 2-6/ α 2-6 linkages (Fig. 1C).

3.3. Identification of sialylated *N*-glycan linkage isomers by RT shift in EIC

The EIC was obtained by plotting the intensity detected at selected *m/z* values corresponding to the sample. The *N*-glycans obtained from mutant by extraction in mono-, di-, and tri-sialylated *N*-glycans at corresponding *m/z* values (calculated *m/z* = 1149.4668, 1295.0145, and 1623.1283, respectively) were investigated, and identified as nos. 12, 20, and 26, respectively (Fig. 2). The EIC in mutant contained multiple peaks with identical *m/z* values, suggesting the presence of their isomers. *N*-glycans of mutant (containing α 2-3 or α 2-6 linkages), α 2-3 neuraminidase-treated mutant

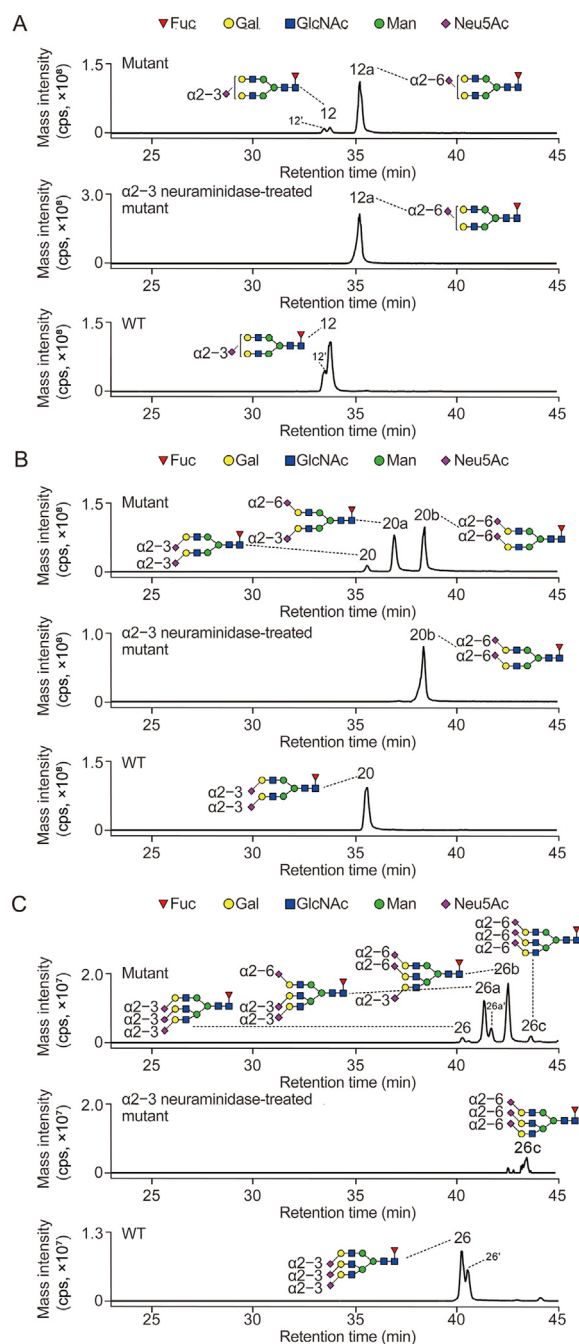


Fig. 2. Extracted ion chromatogram (EIC) of the proA-labeled *N*-glycans. The *N*-glycans from (top) mutant, (middle) α 2-3 neuraminidase-treated mutant, and (bottom) wild-type (WT) at (A) calculated $m/z = 1149.4668$ corresponding to peak nos. 12 and 12a, (B) calculated $m/z = 1295.0145$ corresponding to peak nos. 20, 20a, and 20b, and (C) calculated $m/z = 1623.1283$ corresponding to peak nos. 26, 26a, 26b, and 26c were used. The peak numbers are shown in Table S1. Fuc: fucose; Gal: galactose; GlcNAc: *N*-acetylglucosamine; Man: mannose; Neu5Ac: *N*-acetylneuraminic acid.

(containing only α 2-6 linkages), and WT (containing only α 2-3 linkages) were compared.

No. 12 from the mutant was identified with two distinct peaks at 33.9 (peak no. 12) and 35.3 min (peak no. 12a), and peak no. 12a was abundant in mutant (Fig. 2A). The di-sialylated *N*-glycan as no. 20 from the mutant contains three individual peaks as nos. 20 at 35.7 min, 20a at 37.0 min, and 20b at 38.5 min (Fig. 2B). The peak nos. 20a and 20b were dominant, and the most abundant peak was no. 20b in the mutant. These isomers were distinguished based on

RT in the HILIC column, and the sialylated *N*-glycan with α 2-3 linkage is eluted earlier than that of α 2-6 linkage [34]. Additionally, the MS/MS spectrum of each isomer peak was investigated in Section 3.2, and peak nos. 20, 20a, and 20b corresponded to those in Figs. 1A (α 2-3/ α 2-3 linkages), B (α 2-3/ α 2-6 linkages), and C (α 2-6/ α 2-6 linkages), respectively. Thus, the separation of peaks was caused by sialyl-linkage isomers in EIC, and the peaks from the mutant were determined as α 2-3/ α 2-3 (peak no. 20), α 2-3/ α 2-6 (peak no. 20a), and α 2-6/ α 2-6 linkages (peak no. 20b). Similarly, peak nos. 12 and 12a were α 2-3 and α 2-6 linkages, respectively. The *N*-glycan peaks obtained from tri-sialylated *N*-glycans in mutant were separated into four peaks on different RT as follows: peak nos. 26 at 40.3 min (α 2-3/ α 2-3/ α 2-3), 26a at 41.4 min (α 2-3/ α 2-3/ α 2-6), 26b at 42.6 min (α 2-3/ α 2-6/ α 2-6), and 26c at 43.7 min (α 2-6/ α 2-6/ α 2-6) (Fig. 2C). The abundant peaks were nos. 26a and 26b, but peak no. 26c which contained only α 2-6 linkages showed a significantly low intensity compared to mono- (peak no. 12a) and di-sialylation (peak no. 20b).

The peaks of EIC were further confirmed by *N*-glycans from α 2-3 neuraminidase-treated mutant comprised of only α 2-6 linkages and WT comprised of only α 2-3 linkages. The EIC of no. 12 (Fig. 2A) from α 2-3 neuraminidase-treated mutant showed a clear single peak at the same RT as peak no. 12a in the mutant, and peak no. 12 disappeared, converting from mono- to non-sialylation. The peak from WT was detected at 33.9 min as peak no. 12 in mutant. Peak no. 20b in α 2-3 neuraminidase-treated mutant and peak no. 20 in WT were obtained in EIC (Fig. 2B). As the elution order of HILIC depended on the number of α 2-6-linked sialic acids and peak no. 20a disappeared in α 2-3 neuraminidase-treated mutant, peak no. 20a of mutant was identified with α 2-3/ α 2-6 linkages. The EIC of tri-sialylated *N*-glycan (Fig. 2C) from α 2-3 neuraminidase-treated mutant and WT were identified at peak nos. 26c and 26, respectively.

The mono- and di-sialylated *N*-glycans with bi-antennary structure in Figs. 2A and B showed an RT shift by sialyl-linkage isomers of 1.4 min between α 2-3 and α 2-6 linkages (peak nos. 12 and 12a), 1.3 min between α 2-3/ α 2-3 and α 2-3/ α 2-6 linkages (peak nos. 20 and 20a), and 1.5 min between α 2-3/ α 2-6 and α 2-6/ α 2-6 linkages (peak nos. 20a and 20b). Meanwhile, the tri-sialylated *N*-glycans with tri-antennary structure in Fig. 2C showed an RT shift by sialyl-linkage isomers of 1.1 min between α 2-3/ α 2-3/ α 2-3 and α 2-3/ α 2-3/ α 2-6 linkages (peak nos. 26 and 26a), 1.2 min between α 2-3/ α 2-3/ α 2-6 and α 2-3/ α 2-6/ α 2-6 linkages (peak nos. 26a and 26b), and 1.1 min between α 2-3/ α 2-6/ α 2-6 and α 2-6/ α 2-6/ α 2-6 linkages (peak nos. 26b and 26c). These results indicated that HILIC column provided sufficient specificity for sialylated *N*-glycans with α 2-3 and α 2-6 linkage(s) and the RT shift (1.3–1.5 min) of linkage isomers in bi-antennary structures was slightly longer than that (1.1–1.2 min) in tri-antennary structures.

The *N*-glycan analysis by the HILIC column has high resolution and selectivity for isomers, including sialyl-linkage and their structures [35]. The peak pattern of nos. 12 and 12' from WT and mutant was partially separated (Fig. 2A), and this pattern was reported as the separation of sialic acid positional isomers [19]. Moreover, peak nos. 26 and 26', and 26a and 26a' from WT and mutant were observed with doublets (Fig. 2C), and these should be confirmed with further studies including the determination of their branch structures.

3.4. L_n/N_n value in MS/MS spectra

The L_n/N_n value was calculated as $([GlcNAc] \text{ ions intensity} / [Neu5Ac] \text{ ions intensity}) \times (\eta_{[Neu5Ac]} / \eta_{[GlcNAc]})$ in the MS/MS spectra using a previously reported method [21]. This ratio was obtained based on the tendency of oxonium ions to be intensity derived from

Neu5Ac (m/z 274.0921 + 292.1027) and GlcNAc (m/z 204.0867 + 366.1395) depending on sialyl-linkage isomers in MS/MS spectra. As oxonium ions were affected by the number of monosaccharides, the ratio was divided by the compensation factor as the number of GlcNAc and Neu5Ac.

The calculated Ln/Nn values were as follows: 0.7 calculated from $[(3.6 \times 10^5 + 5.2 \times 10^5)/(3.0 \times 10^5 + 3.2 \times 10^5) \times 2/4]$, 1.3 calculated from $[(4.4 \times 10^6 + 8.0 \times 10^6)/(2.1 \times 10^6 + 2.6 \times 10^6) \times 2/4]$, and 3.0 calculated from $[(3.6 \times 10^6 + 8.3 \times 10^6)/(8.6 \times 10^5 + 1.1 \times 10^6) \times 2/4]$ (Figs. 1A–C, respectively). The Ln/Nn values of the remaining sialylated *N*-glycans were obtained with the same methods, and the presented ratio was the average value ($n = 3$) of each isomer in the MS/MS spectra with error bars for standard deviation. The precursor ions of MS/MS spectra were predominantly observed in 2+ charged states, and tetra-antennary *N*-glycans were used in 3+ charged states. In this study, the Ln/Nn values of the sialylated *N*-glycans containing only α 2-3 linkage(s) ranged from 0.5 to 1.3, whereas the ratios of those containing only α 2-6 linkage(s) ranged from 1.6 to 4.7 (Fig. 3). The sialylated *N*-glycans containing both α 2-3 and α 2-6 linkages overlapped (range 1.0–2.2).

Nos. 16 and 16a had the highest value (1.3 and 4.7) in the α 2-3 and α 2-6 linkage groups, respectively, and the *N*-glycan composition of those was of a tri-antennary mono-sialylated *N*-glycan. The Ln/Nn values of sialylated *N*-glycans modified with Neu5Gc corresponding to nos. 14 and 14a and *O*-acetylated Neu5Ac corresponding to nos. 7, 9, 9a, 18, 18a, 18b, 19, 19a, 19b, 21, 25, 25a, and 25b were also different, depending on sialyl-linkage isomers (Fig. 3). In these cases, $[\text{Neu5Gc-H}_2\text{O}]^+$ and $[\text{Neu5Gc}]^+$ ions at calculated m/z 290.0870 and 308.0976, and $[\text{Neu5Ac-OAc-H}_2\text{O}]^+$ and $[\text{Neu5Ac-OAc}]^+$ ions at calculated m/z 316.1027 and 334.1133 were used for calculation.

No. 19a as bi-antennary di-sialylated *N*-glycan containing Neu5Ac and *O*-acetylated Neu5Ac with α 2-3 and α 2-6 linkages showed partially two peaks at 33.4 and 33.7 min (Fig. S4A). In each MS/MS spectrum, the relative intensity of Neu5Ac ions (22.1% and 23.7%) was higher than that of *O*-acetylated Neu5Ac ions (9.2% and 11.2%) about twice at 33.4 min (Fig. S4B), while *O*-acetylated Neu5Ac ions (22.3% and 30.7%) were higher than Neu5Ac ions (9.0% and 10.2%) at 33.7 min (Fig. S4C). It was considered as a positional isomer of *O*-acetylation, which indicated *O*-acetylated Neu5Ac with α 2-6 linkage (Fig. S4B) or α 2-3 linkage (Fig. S4C). In the sialylated *N*-glycans containing both α 2-3 and α 2-6 linkages, modification of *O*-

acetylation in Neu5Ac with α 2-6 linkage was eluted before that with α 2-3 linkage (Fig. S4A). However these two isomers were shown to be similar Ln/Nn values as 1.2 and 1.1 within the range of both α 2-3 and α 2-6 linkages group.

In order to distinguish α 2-3 and α 2-6 linkages effectively, the distribution was identified by modifying calculation of Ln/Nn value. The HexNAcHex ion (m/z 366.1395) was used as GlcNAc ion intensity instead of HexNAcHex ion + HexNAc ion (m/z 204.0867) (Fig. S5A) of Ln/Nn value, because the relative intensity of HexNAc ion was not clearly changed according to linkage isomers. The calculation was modified as $[\text{GlcNAc ion intensity (} m/z \text{ 366.1395) / Neu5Ac ions intensity (} m/z \text{ 274.0921 + 292.1027)}] \times (\eta_{[\text{Neu5Ac}]} / \eta_{[\text{GlcNAc}]})$. The ratios were ranged in only α 2-3 (0.2–1.1), both α 2-3 and α 2-6 (0.6–1.5), and only α 2-6 linkages (0.9–4.1), and the ranges of only α 2-3 and only α 2-6 linkage(s) were not distinctive compared to Ln/Nn value (Fig. S5B). Additionally, the ProA-HexNAc ion (m/z 441.2708) was utilized for GlcNAc ion intensity, and the compensation factor of GlcNAc was not considered due to ProA on reducing end of *N*-glycans. The ratios were calculated by $[\text{Neu5Ac ions intensity (} m/z \text{ 274.0921 + 292.1027) / ProA-HexNAc ion intensity (} m/z \text{ 441.2708)}] \times 1/\eta_{[\text{Neu5Ac}]}$. The range of only α 2-3 (5.9–12.5, except for one value as 3.6), both α 2-3 and α 2-6 (3.7–9.1), and only α 2-6 linkages (1.5–5.1) were presented (Fig. S5C). These ranges were separated in only α 2-3 and only α 2-6 linkages. However, the ratios showed relatively high standard deviation compared to Ln/Nn value due to variation of ProA-HexNAc ion intensity. Thus, the Ln/Nn value was determined as described above in this study.

The previously reported Ln/Nn values in *N*-glycopeptides were investigated, and those indicated ranges of 0.45–0.69 and 0.86–1.53 for α 2-3 and α 2-6 linkages, respectively, at 30% NCE in HCD [21]. Another study reported that Ln/Nn values of glycopeptides had two distinct distributions, but they were closer to each other than expected [36]. Different strategies to distinguish sialyl-linkage isomers based on fragmentation were analyzed by MS³ spectra of $[\text{HexNAcHexNeu5Ac}]^+$ ions at m/z 657.2349 in *N*-glycopeptides and *N*-glycans [22]. However, the efficiency of Ln/Nn value for ProA-labeled *N*-glycans used in this study has not been reported yet, and we investigated the accuracy and usefulness of Ln/Nn for application to identification of *N*-glycans. The ProA-labeled *N*-glycans prepared from fetuin (Sigma-Aldrich), which is a well-reported glycoprotein, were investigated with the same method

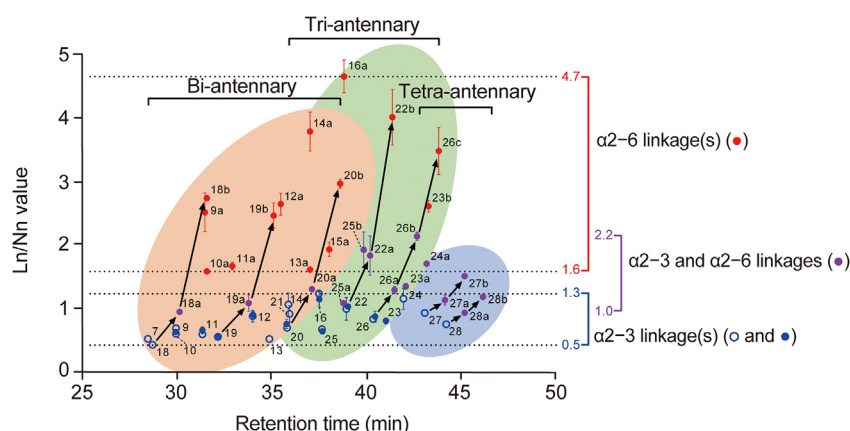


Fig. 3. Scatter plot of the *N*-acetylglucosamine ion to the sialic acid ions ratio (Ln/Nn) versus retention time. The Ln/Nn values are presented with average value in procainamide-labeled sialylated *N*-glycans, and the error bars show standard deviation ($n = 3$). Mono- (nos. 7–16), di- (nos. 17–24), tri- (nos. 25–27), and tetra- (no. 28) sialylation are presented. The arrows indicate linkage isomers with the same *N*-glycan composition. ○: sialylated *N*-glycans with only α 2-3 linkage(s) from wild-type (WT); ●: sialylated *N*-glycans with only α 2-3 linkage(s) from mutant; ●: sialylated *N*-glycans with both α 2-3 and α 2-6 linkages from mutant; ●: sialylated *N*-glycans with only α 2-6 linkage(s) from mutant; orange ellipse: bi-antennary structures; green ellipse: tri-antennary structures; blue ellipse: tetra-antennary structures.

used in this study. The Ln/Nn values of 12 sialyl-linkage isomers of fetuin were obtained, as follows (Table S2). Ln/Nn values with only α 2-3 linkage(s) in MS/MS spectra were 0.9 (bi-antennary mono-sialylation), 0.7 (bi-antennary di-sialylation), 1.1 (tri-antennary di-sialylation), and 1.1 (tri-antennary tri-sialylation), which were included in the range of only α 2-3 linkages indicated in this study. The Ln/Nn values of the other sialylated *N*-glycans in fetuin were 1.3 (bi-antennary di-sialylation), 1.8 (tri-antennary di-sialylation), 1.4 (tri-antennary tri-sialylation), and 2.1 (tri-antennary tri-sialylation) in both α 2-3 and α 2-6 linkages, and 2.7 (bi-antennary mono-sialylation), 2.8 (bi-antennary di-sialylation), 3.3 (tri-antennary di-sialylation), and 3.2 (tri-antennary tri-sialylation) in only α 2-6 linkage(s), which were also involved in the range of both α 2-3 and α 2-6 linkages and only α 2-6 linkages, respectively. These results indicated that the present results for Ln/Nn values are useful and applicable for *N*-glycan linkage isomers.

The Ln/Nn values of the sialylated *N*-glycans containing only α 2-3 linkages with an average value of 0.8 (0.5–1.3), both α 2-3 and α 2-6 linkages with an average value of 1.4 (1.0–2.2), and only α 2-6 linkages with an average value of 2.8 (1.6–4.7) (Fig. 3) suggested that the Ln/Nn value is useful in distinguishing among linkage isomers, although ratios of glycopeptides and *N*-glycans are rather different values.

3.5. Scatter plot of Ln/Nn value versus RT

The α 2-3 and α 2-6 linkages were determined by combining RT (x-axis) in HILIC column and Ln/Nn value (y-axis) in MS/MS spectra, and those were summarized with scatter plot obtained by the present results (Fig. 3). With the increase of α 2-6 linkage in linkage isomer with the same *N*-glycan composition, the RT and Ln/Nn value were increased. According to the increased values in x-axis, the identified *N*-glycans also increased from bi- to tetra-antennary structures (Fig. 3). The bi-antennary di-sialylated *N*-glycans modified with *O*-acetylation showed different RT and Ln/Nn such as nos. 18 (28.6 min and 0.5), 18a (30.0 min and 1.0), 18b (31.4 min and 2.8), 19 (32.1 min and 0.6), 19a (33.7 min and 1.1), and 19b (35.0 min and 2.5). The bi-antennary di-sialylated *N*-glycan was identified with nos. 20 (35.7 min and 0.8), 20a (37.0 min and 1.4), and 20b (38.5 min and 3.0). Those values were indicated as nos. 18, 19, and 20 for α 2-3/ α 2-3 linkages; nos. 18a, 19a, and 20a for α 2-3/ α 2-6 linkages; and nos. 18b, 19b, and 20b for α 2-6/ α 2-6 linkages. The tri-antennary structures were also detected with di-sialylation such as nos. 22 (38.9 min and 1.1), 22a (40.1 min and 1.9), 22b (41.3 min and 4.1), and tri-sialylation such as nos. 26 (40.3 min and 0.9), 26a (41.4 min and 1.3), 26b (42.6 min and 2.2), and 26c (43.7 min and 3.5). The sialyl-linkage isomers were determined with nos. 22, 22a, and 22b for α 2-3/ α 2-3, α 2-3/ α 2-6, and α 2-6/ α 2-6 linkages, respectively, and nos. 26, 26a, 26b, and 26c for α 2-3/ α 2-3/ α 2-3, α 2-3/ α 2-3/ α 2-6, α 2-3/ α 2-6/ α 2-6, and α 2-6/ α 2-6/ α 2-6 linkages, respectively.

The boundary of tetra-antennary *N*-glycans demonstrated relatively small changes in the y-axis compared to that of bi- and tri-antennary structures, but those were not detected with sialylated *N*-glycans containing only α 2-6 linkages. The values in tetra-antennary tri-sialylation as nos. 27 (43.0 min and 1.0), 27a (44.0 min and 1.2), and 27b (45.1 min and 1.6) were obtained. The tetra-sialylated *N*-glycans were shown to have a similar tendency as nos. 28 (44.1 min and 0.8), 28a (45.1 min and 1.0), and 28b (46.0 min and 1.2). Nos. 27 and 28 contained only α 2-3 linkages and nos. 27a, 27b, 28a, and 28b were involved in both α 2-3 and α 2-6 linkages.

3.6. Identification of *N*-glycans

N-glycan profiles from WT and mutant were detected by TIC using LC-ESI-MS/MS (Fig. S6 and Table S3), and their structures

were confirmed using MS/MS fragmentations, indicated in Sections 3.1 to 3.4. The previous report identified eight *N*-glycans (nos. 1, 3, 5, 12, 16, 19, 22, and 26) [7], and the others included linkage isomers that were identified in this study. The repeatability and intermediate precision of the experiments were evaluated by RSD, and relative quantity of three major glycans (nos. 3, 12, and 20) were used for RSD. The repeatability and intermediate precision were less than 3% ($n = 3$) and 7% ($n = 3$), respectively (Table S4).

The 28 *N*-glycan peaks from WT (Fig. S6A) and the 65 *N*-glycan peaks from mutant (non- and mono-sialylation (Fig. S6B; 26 *N*-glycan peaks, nos. 1–16) and di-, tri-, and tetra-sialylation (Fig. S6C; 39 *N*-glycan peaks, nos. 17–28)) were identified.

As indicated in Table S1, *N*-glycans were numbered by the *N*-glycan structures in the corresponding peaks, and were summarized depending on sialylation degree as non- (peak nos. 1–6), mono- (peak nos. 7–16), di- (peak nos. 17–24), tri- (peak nos. 25–27), and tetra-sialylation (peak no. 28). The sialyl-linkage isomers were categorized according to the number of α 2-6 linkages in corresponding *N*-glycan, such as 0 (absence of α 2-6 linkage), 1 (1 α 2-6 linkage), 2 (2 α 2-6 linkages), and 3 (3 α 2-6 linkages). All detected *N*-glycans were eluted in a range of RT from 23 to 47 min. The obtained *N*-glycan peaks from mutant in TIC were heterogeneous compared to that of WT, caused by sialyl-linkage isomers in mutant (Fig. S6). The peak nos. 5 and 12 as non- and mono-sialylated *N*-glycan were identified at 31.8 and 33.9 min in both WT and mutant (Figs. S6A and B), but the isomer peak as no. 12a was only detected in mutant. The *N*-glycan peaks with the same composition were separated into several peaks such as peak nos. 20, 20a, and 20b at 35.7, 37.0, and 38.5 min, respectively and peak nos. 26, 26a, 26b, and 26c at 40.3, 41.4, 42.6, and 43.7 min, respectively (Fig. S6C). The *N*-glycan peaks with the same peak numbers between WT and mutant were observed at the exactly same RT (Fig. S6), and the isomer peaks with same *N*-glycan composition showed an RT shift in mutant (Figs. S6B and C). In Figs. S6A and B, the peak nos. 7, 9, and 12 (data not shown) eluted at 28.3, 29.8, and 33.9 min were bi-antennary mono-sialylated *N*-glycans with di-*O*-acetylation, mono-*O*-acetylation, and non-*O*-acetylation, respectively (Fig. S2). The RT of sialylated *N*-glycans containing *O*-acetylation were significantly shorter than that modified with non-*O*-acetylation due to hydrophobicity of *O*-acetyl group [5].

The labeling efficiency (%) was estimated as (EIC area of unlabeled *N*-glycan/EIC area of ProA-labeled *N*-glycan) $\times$ 100, using three major *N*-glycans (nos. 3, 12, and 20 in Table S1). The labeling efficiency was less than 0.3% obtained from WT (no. 3, 0.1%; no. 12, 0.2%; no. 20, 0.1%) and mutant (no. 3, 0.3%; no. 12, 0.2%; no. 20, 0.1%).

3.7. Quantification of *N*-glycans

The quantities of each *N*-glycan were obtained by the EIC area relative to the sum of the EIC area for all of the *N*-glycans, because the *N*-glycan peaks can be individually quantified using EIC [35]. Thus, the quantification by EIC area is more reliable and accurate. The previous report quantified 8 *N*-glycans (nos. 1, 3, 5, 12, 16, 19, 22, and 26) [7], and the others including linkage isomers were identified in this study. The total number of *N*-glycans was 26 and 45 except for those measured as trace amounts (<0.1%) in relative quantity from WT and mutant, respectively. The relative quantification of all identified *N*-glycans from WT and mutant was obtained and summarized in Table S1, and those in mutant are indicated as follows.

Non-sialylated *N*-glycans consist of six *N*-glycans. Sum of no. 1 (relative quantity of 11.5%), no. 3 (20.7%), and no. 5 (6.3%) was

occupied with 38.5% and the total relative quantities of non-sialylated *N*-glycans were 41.2% in mutant. The mono-sialylated *N*-glycans were composed of 15 *N*-glycans including sialyl-linkage isomers. Of these, sialylations were modified with *O*-acetylation (nos. 9 and 9a, 0.4%) and Neu5Gc (no. 14a, 0.3%). The most abundant *N*-glycan was no. 12a containing 11.5%, and the total relative quantities of mono-sialylated *N*-glycans were 25.4%. The di-sialylated *N*-glycans were detected with 15 *N*-glycans, and modification of *O*-acetylation was identified with 6 *N*-glycans (2.6%). The *N*-glycans having the same composition as no. 20 were predominantly occupied with 22.1% in di-sialylation. The total relative quantities of di-sialylated *N*-glycans were 28.4%. The 8 *N*-glycans with tri-sialylation were detected, and nos. 25a and 25b contained modification of *O*-acetylation (0.5%). The total relative quantities with tri-sialylation were 4.8%. The *N*-glycan with tetra-sialylation was identified as one *N*-glycan composition occupied with 0.2%.

The increase of the sialylation with overexpression of $\alpha 2$ -3 or $\alpha 2$ -6 ST, and the increase of the antennary structures with overexpression of GnT-IV and GnT-V, which facilitate ST activity, in CHO cells have been previously reported [3]. Meanwhile, the distributions of sialylation of recombinant human erythropoietin produced in CHO cells with co-overexpression of GnT-IV, GnT-V, and $\alpha 2$ -6 ST were decreased by 4.8%, 9.1%, and 9.5% in non-, mono-, and di-sialylated *N*-glycans, respectively and were increased by 16.3% and 7.1% in tri- and tetra-sialylated *N*-glycans, respectively, as analyzed by MALDI-MS [37]. Additionally, recombinant human interferon- γ in CHO cells co-overexpressed with GnT-V and $\alpha 2$ -6 ST were analyzed by anion-exchange high performance liquid chromatography, and the average number of sialic acids in *N*-glycans was increased from 1.33 to 2.34 [38].

In this study, 26 and 45 *N*-glycans in WT and mutant were composed of 1) 6 (49.6%) and 6 (41.2%) non-, 2) 10 (28.1%) and 15 (25.4%) mono-, 3) 6 (19.8%) and 15 (28.4%) di-, 4) 3 (2.4%) and 8 (4.8%) tri-, and 5) 1 (0.1%) and 1 (0.2%) tetra-sialylation, respectively. These results indicated that non- and mono-sialylated *N*-glycans were decreased while di-, tri-, and tetra-sialylated *N*-glycans were increased in mutant, compared to those of WT. Additionally, the relative quantities of *O*-acetylation in sialic acid were quantified as 3.9% (WT) and 3.5% (mutant), and these can be affected to therapeutic glycoproteins such as influence of protein solubility by reducing Neu5Ac hydrophilicity as well as increase of half-life by inhibiting neuraminidase activity [5].

3.8. Quantification of sialylated *N*-glycan linkage isomers

The proportion of sialyl-linkage isomers is represented in a bar graph according to sialylation degree in WT and mutant (Fig. 4). All sialylated *N*-glycans were observed for $\alpha 2$ -3 linkages in WT. In mutant, the ratio of mono-sialylated *N*-glycans was 11:89 (relative quantity of 2.9% and 22.5%) for $\alpha 2$ -3 and $\alpha 2$ -6 linkages, and those of di-sialylated *N*-glycans were 6:49:45 (relative quantity of 1.7%, 13.8%, and 12.9%) for $\alpha 2$ -3/ $\alpha 2$ -3, $\alpha 2$ -3/ $\alpha 2$ -6, and $\alpha 2$ -6/ $\alpha 2$ -6 linkages. These results showed a higher ratio of $\alpha 2$ -6 linkages than that of $\alpha 2$ -3 linkages. While the tri-sialylated *N*-glycans were quantified as 0.2%, 2.1%, 2.3%, and 0.2% for $\alpha 2$ -3/ $\alpha 2$ -3/ $\alpha 2$ -3, $\alpha 2$ -3/ $\alpha 2$ -3/ $\alpha 2$ -6, $\alpha 2$ -3/ $\alpha 2$ -6/ $\alpha 2$ -6, and $\alpha 2$ -6/ $\alpha 2$ -6/ $\alpha 2$ -6 linkages, respectively and their ratio was 4:44:48:4, those were shown to be similar between $\alpha 2$ -3 and $\alpha 2$ -6 linkages. The isomers of tetra-sialylated *N*-glycans were identified with $\alpha 2$ -3/ $\alpha 2$ -3/ $\alpha 2$ -3/ $\alpha 2$ -6 linkages (relative quantity of 0.2%).

The proportion of sialylated *N*-glycans with $\alpha 2$ -3 and $\alpha 2$ -6 linkages from mutant contained the higher contents of $\alpha 2$ -6 linkages than those of $\alpha 2$ -3 linkages in mono- and di-sialylation. The tri-sialylated *N*-glycans were similar values between $\alpha 2$ -3 and $\alpha 2$ -6

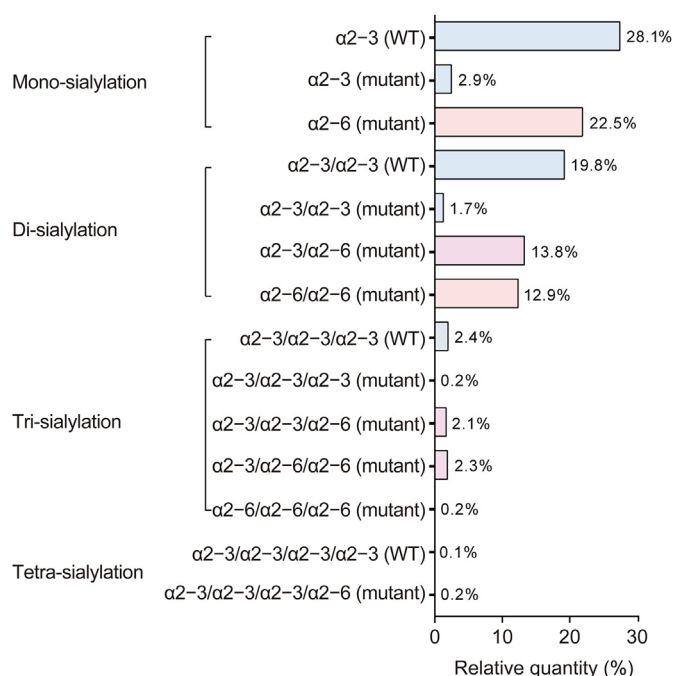


Fig. 4. Sum of relative quantities of $\alpha 2$ -3 and $\alpha 2$ -6 linkages in procainamide-labeled sialylated *N*-glycans. The mono-, di-, tri-, and tetra-sialylated *N*-glycan isomers in the wild-type (WT) and mutant.

linkages. However, the isomers with di-sialylation were shown to have different tendency as nos. 21–24 (0.3%, 2.8%, 0.7% for $\alpha 2$ -3/ $\alpha 2$ -3, $\alpha 2$ -3/ $\alpha 2$ -6, $\alpha 2$ -6/ $\alpha 2$ -6) with tri-antennary structures and nos. 17–20 (1.4%, 11.0%, 12.2% for $\alpha 2$ -3/ $\alpha 2$ -3, $\alpha 2$ -3/ $\alpha 2$ -6, $\alpha 2$ -6/ $\alpha 2$ -6) with bi-antennary structures (Table S1). Thus, the proportion of $\alpha 2$ -3 and $\alpha 2$ -6 linkages was affected by the antennary structures.

Reports have described the antenna specificity of $\alpha 2$ -3 and $\alpha 2$ -6 ST. The tri- and tetra-antennary *N*-glycans are more frequently observed with $\alpha 2$ -3-linked sialic acids whereas the bi-antennary *N*-glycans are dominant with $\alpha 2$ -6-linked sialic acids [38]. CHO cells in mutant conditions had both $\alpha 2$ -3 and $\alpha 2$ -6 ST, and these were mutually complementary to sialylation of *N*-glycans in CTLA4-Ig. Although the ratio of $\alpha 2$ -3- and $\alpha 2$ -6-linked sialic acids changed depending on antennary structures, their total ratios were 28.5% and 71.5% in mutant, respectively. The ratio of $\alpha 2$ -3-linked sialic acids was calculated as follows: [(sum of each relative quantity of sialylated *N*-glycans $\times$ the number of $\alpha 2$ -3-linked sialic acids)/(sum of each relative quantity of sialylated *N*-glycans $\times$ the number of sialic acids)] $\times$ 100.

The interferon- γ produced in CHO cell co-overexpressing GnT-V and rat $\alpha 2$ -6 ST were reported that proportion of $\alpha 2$ -3- and $\alpha 2$ -6-linked sialic acids were determined as 17.4% and 82.6% by $\alpha 2$ -3 neuraminidase treatment [38]. The distribution of $\alpha 2$ -3- and $\alpha 2$ -6-linked sialic acids was also identified with 15% and 85% in trastuzumab co-overexpressed with galactosyltransferase and $\alpha 2$ -6 ST, which were determined by $\alpha 2$ -3 neuraminidase treatment [39].

Meanwhile, the sialyl-linkage isomers can be associated with the progression of some diseases. The cell surfaces on human malignant cells frequently have increased $\alpha 2$ -6-linked sialic acids, whereas upregulation of $\alpha 2$ -3-linked sialic acids in sialyl Lewis X and a motif promoted metastasis of cancer cells [16,21]. A shift toward $\alpha 2$ -3- from $\alpha 2$ -6-linked sialic acids pathologically occurred in chondrocytes [40]. Furthermore, the interaction of glycan and apoptosis-inducing galectin proteins was inhibited by $\alpha 2$ -6-linked sialic acids, to promote cell survival [16]. The avian and human

influenza viruses preferentially recognize α 2-3- and α 2-6-linked sialic acids, respectively, as a cellular receptor [41], and these are related to viral tissue tropism and zoonotic spread [21].

4. Conclusion

In this study, sialylated *N*-glycan isomers with α 2-3 and α 2-6 linkages in CTLA4-Igs were distinctively and successfully identified and quantified by LC-ESI-MS/MS with HILIC columns by enhancing ionization efficiency with ProA, relative intensities of oxonium ions (Ln/Nn value) in MS/MS spectra, and separation of mass peaks due to RT shift in EIC. The present study obtained the following results: 1) the relative intensities of two sialic acid ions in α 2-6 linkage were decreased compared to those in α 2-3 linkage; however, that of HexNAcHex ion in α 2-6 linkage was increased compared to that in α 2-3 linkage; 2) the intensity of oxonium ions was used to calculate the Ln/Nn value, and the values showed the ranges for sialylated *N*-glycans containing only α 2-3 linkage(s) group (0.5–1.3), only α 2-6 linkage(s) group (1.6–4.7), and both α 2-3 and α 2-6 linkages group (1.0–2.2); 3) mono-, di-, and tri-sialylated *N*-glycans showed a different RT between α 2-3 and α 2-6 linkage(s), and RT shift (1.3–1.5 min) in mono- and bi-antennary structures was slightly different from those (1.1–1.2 min) in tri- and tetra-antennary structures; and 4) *N*-glycan analysis by HILIC columns had sufficient resolution and selectivity for α 2-3 and α 2-6 linkages. Consequently, the *N*-glycans from WT were obtained as 20 sialylated *N*-glycans (50.4%) with only α 2-3 linkages, but 39 sialylated *N*-glycan isomers (58.8%) with only α 2-3 (10 *N*-glycans; 4.8%), both α 2-3 and α 2-6 (14; 18.4%), and only α 2-6 (15; 35.6%) linkages were obtained in mutant. These results are consistent with those by α 2-3 neuraminidase-treated *N*-glycans, and all of the present results were summarized as a scatter plot of Ln/Nn (*y*-axis) versus RT (*x*-axis). Additionally, the quantification of *N*-glycans was determined by EIC area for all observed ionization states of each identified peak. This is the first study to demonstrate and suggest a plot of Ln/Nn versus RT of the LC-MS/MS-obtained *N*-glycan mass peaks in therapeutic glycoprotein to distinguish among sialylated *N*-glycan isomers with α 2-3 and/or α 2-6 linkage(s) without additional sialic acid derivatization.

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Declaration of competing interest

The authors declare that there are no conflicts of interest.

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Appendix A. Supplementary data

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