N-Glycan structures from the major glycoproteins of pigeon egg white: Predominance of terminal Galα(1-4)Gal

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Summary

N-Glycans from major glycoproteins of pigeon egg white (ovotransferrin, ovomucoid, ovalubumins) were enzymatically released and were reductively aminated with 2-aminopyridine, separated, and structurally characterized by mass spectrometry and a three-dimensional mapping technique using three different columns of high-performance liquid chromatography (HPLC) [Takahashi, et al. (1995) Anal. Biochem. 226, 139—146]. Twenty five major N-glycan structures, all of them hitherto unknown, were identified as pyridylamino derivatives. Of these, thirteen were neutral, ten were mono-sialyl and two were di-sialyl oligosaccharides. All N-glycans contain from one to four Gal(1,4)Galβ(1,4) sequences at the nonreducing terminal positions and are devoid of fucose residues. N-Acetylneuraminic acids were α(2,6)-linked only to β-galactose. The HPLC profiles of the N-glycans from four different glycoproteins were qualitatively very similar to each other, but not identical in the peak distributions. Mono-sialyl glycans were most abundant in all four glycoproteins, followed by neutral glycans. Di-sialyl glycans were lowest in ovotransferrin, and highest in ovomucoid. Triantennary structures with bisecting GlcNAc were predominant in ovotransferrin, and tetraantennary (with and without bisecting GlcNAc-containing) structures were predominant in other glycoproteins. Pentaantennary structures (with a sialic acid and without bisecting GlcNAc residue) were also found in small quantities in all four glycoproteins. In contrast to the chicken egg white counterparts, which contain mostly high-mannose and hybrid types, all N-glycan structures in the major pigeon egg white glycoproteins are complex type.
Introduction

The presence of galabiose (Galα4Gal) sequence in N-glycans in any animal is only rarely noted. The only documented presence of such a sequence in N-glycan was that found in turtle dove ovomucoid (1,2). The only other recorded presence of galabiose sequence were both in the O-glycosides found in the Chinese swiftlet nest (3) and in an insect cell line *Mamestra brassicae* clone SPCMb-92-C6 (4). As shown in the preceding paper (5)[Suzuki et al, M1:01379], we have demonstrated that galabiose sequence is abundantly present in pigeon egg white glycoproteins, and it always occupies the terminal positions of the multiantennary structures. In the major N-glycans from the pigeon ovalbumin, no sialylation is found on the branch which contains the galabiose sequence. In contrast, chicken ovalbumin contained mostly high-mannose type and hybrid types (6,7) while chicken ovomucoid contains mostly galactose-less complex-type (8). The preceding paper also notes that, in contrast to the chicken and other avian egg whites hitherto investigated, the N-glycans in pigeon egg white glycoproteins seem to be more “mature”, containing more of the multiantennary complex type structures. In view of these unique structural features, we felt that detailed structural studies were in order, not only to understand the underlying evolutionary significance but to be better able to utilize these unique features for biomedical applications. We have undertaken such studies, employing the powerful methodology of the 3-D mapping (9-12) as well as various forms of mass spectroscopy. We have elucidated the fine structures of the major oligosaccharide of ovotransferrin, ovomucoid, and ovalbumins from pigeon egg white, the results of which are reported here. In all, 25 different structures were identified, all of which were hitherto unknown structures.
EXPERIMENTAL PROCEDURES

MATERIALS

Enzymes—Glycoamidase A (also known as glycopeptidase A) from sweet almond (13), β-galactosidase, and β-N-acetylhexosaminidase from jack bean were purchased from Seikagaku Kogyo Co. (Tokyo, Japan). α-Galactosidase from coffee bean was purchased from Oxford GlycoSciences, Inc., (Bedford, MA). Sialidase from Arthrobacter ureafaciens was obtained from Nacalai Tesque (Kyoto, Japan). Trypsin, chymotrypsin and pronase were from Sigma Chem. Co. (St. Louis, MO).

Pigeon egg white glycoproteins—Pigeon eggs were collected from local racing pigeon breeders in the Minneapolis-St. Paul (MN) metropolitan areas. Both egg white and egg yolk (separated manually) were lyophilized and kept at 4°C until use.

Isolation of major glycoproteins from pigeon egg white by HPLC—Lyophilized pigeon egg white (200 mg), was dissolved with 1.5 ml of distilled water and centrifuged to remove insoluble materials. Supernatant was filtered through a 0.45-μm membrane, and samples of typically ca. 20 mg protein were injected on a 21.2 x 250 mm Jupiter RP-HPLC C4 columns (Phenomenex, Torrance, CA). The mobile phases were (A) 0.05% TFA and (B) 90 % CH₃CN in H₂O containing 0.05% TFA. The elution (8 ml/min) was by a linear gradient of 10 to 70% of (B) in (A) developed over 30 min followed by isocratic elution for 10 min. Proteins eluted were detected by A₂₈₀nm. Individual peaks were collected, dialyzed against water in the cold, and then lyophilized.

Reference N-glycans—The pyridylamino (PA) -derivatives of isomalto-oligosaccharides (4–20 glucose residues) and of reference N-glycans (Code Nos². 300.8 and 400.16) were from Seikagaku Kogyo. The following N-glycans were prepared by the known
methods (11): Triantennary with bisecting N-acetylglucosamine residue (Code No. 301.8) and the one of the mono-sialyl 301.8 (Code No. 1A1-301.8) were from nicotinic acetylcholine receptor from *Torpedo californica* (14). A tetraantennary oligosaccharide with bisecting N-acetylglucosamine (Code No. 401.16) and the one of the mono-sialyl 401.16 (Code No. 1A4-401.16) were prepared from peanut agglutinin receptor isolated from human gastric cancer cells (15). One of the mono-sialyl triantennary structures (Code No. 1A1-300.8) was prepared from fetuin tri-sialyl triantennary (Code No. 3A3-300.8), by elimination of two of the sialic acid residues with specific α(2,3)-sialidase digestion. One of the mono-sialyl tetraantennary structures (Code No. 1A4-400.16) was selectively prepared from a neutral tetraantennary compound, Code No. 400.16, using a bovine colostrum α(2,6)-sialyltransferase (16). Structures and Code Nos. of reference compounds used in this study are shown in Table II.

**METHODS**

*Preparation of pyridylaminated N-glycans from pigeon glycoproteins and analysis by 3-D mapping—*PA-oligosaccharides from pigeon ovotransferrin (POT), ovomucoid (POM), ovalbumin (high) (POA(hi)), and ovalbumin (low) (POA(lo)) were prepared as described (11). POT (10 mg), POM (1 mg), POA(hi) (2 mg) and POA(lo) (2 mg), each corresponds to ca. 130 nmol of N-glycans, were used as starting materials. All experimental procedures used in this work, including chromatographic conditions, have been detailed previously (10,11). Briefly, each glycoprotein was proteolysed with chymotrypsin and trypsin mixture, and the proteolysate was further digested with glycoamidase A to release N-glycans. After
removing the peptidic materials, the reducing ends of \( N \)-glycans were derivatized with 2-aminopyridine \(^{17}\).

The mixture of PA-oligosaccharides was separated by HPLC on a diethylaminoethyl (DEAE) column according to the sialic acid content. Each separated fraction was then applied to an octadecylsilica (ODS) HPLC column, and the elution time of each peak was recorded in glucose unit (GU) and plotted on the X-axis. Then, each of the separated \( N \)-glycans was applied to the amide-silica column to separate on the basis of size. Each peak’s elution time was recorded in GU and plotted on the Y-axis. The elution times of a given compound from these two columns (ODS and amide-silica) provide a unique set of coordinates, and the coordinates of the unknown PA-glycan on the 2-D map were compared with those of the authentic reference compounds.

Identification of Structures by 2-D mapping technique—The GU of all unknown \( N \)-glycans were first compared with those of the reference \( N \)-glycans on the 2-D map (containing more than 400 reference \( N \)-glycans). Identification of a sample PA-glycan was confirmed by co-chromatography with a suitable reference \( N \)-glycan on the ODS and amide-silica columns.

For further confirmation, the sample PA-glycan and the reference compound were digested in parallel with one or more exo-glycosidases. At each step of the trimming, the elution positions of the resultant PA-glycans from each were examined for the identity with the expected reference PA-glycan. The trimming and comparison was continued until the products from the sample and the original reference compound became totally coincidental with a known PA-oligosaccharide (trimannosyl core).
Each PA-glycan (50 pmol) isolated on the ODS and amide-silica columns was digested by exoglycosidases (α-sialidase from A. ureafaciens, β-galactosidase and β-N-acetyl-hexosaminidase from jack bean under conditions described previously (10,18). α-Galactosidase from coffee bean (20 mU) was used in 15 µl of 0.1 M citrate-phosphate buffer (pH 6.0) at 37°C for 15 h.

**Permethylolation, FAB- and ESI-Mass spectrometry analysis**—PA-oligosaccharides were permethylated using the NaOH/dimethyl sulfoxide slurry method as described by Dell et al. (19). Fast atom bombardment mass spectrometry (FAB-MS) analyses were performed on an Autospec orthogonal acceleration-time of flight (OA-TOF) mass spectrometer (Micromass, United Kingdom), fitted with a magnet bypass flight tube, and interchangeable FAB source assemblies. For FAB-Mass spectrometry experiments, the fitted cesium ion gun was operated at 26 kV and the source accelerating voltage at 8 kV. The permethyl derivatives of the N-glycans were redissolved in CH$_3$OH for loading onto the probe tip coated with glycerol : m-nitrobenzylalcohol : trifluoroacetic acid (50 : 50 : 1, v/v/v) as matrix. For ESI-Mass spectrometry, the accelerating voltage was maintained at 4 kV which allowed magnet scanning above the mass range of m/z 5000. Hall probe calibration was used. The permethyl derivatives were dissolved in CH$_3$OH and 10 µl aliquots were injected through a Rheodyne loop into the mobile phase (methanol:water:acetic acid, 50:50:1, v:v:v), delivered at a flow rate of 5 µl/min into the ESI source by a syringe pump.

**Linkage analysis by methylation and GC-Mass spectrometry**—For gas chromatography (GC)-electron impact (EI)-Mass spectrometry linkage analysis, partially methylated alditol acetates (PMAA) were prepared from the permethyl derivatives by hydrolysis (2 M trifluoroacetic acid, 121°C, 2 h), reduction (10 mg/ml NaBH$_4$, 25°C, 2 h),
and acetylation (acetic anhydride, 100 °C, 1 h). GC-EI-Mass spectrometry was carried out using a Hewlett-Packard Gas Chromatograph 6890 connected to a HP 5973 Mass Selective Detector. Sample was dissolved in hexane prior to splitless injection into a HP-5MS fused silica capillary column (30 m x 0.25 mm i.d., HP). The column head pressure was maintained at around 8.2 psi to give a constant flow rate of 1 ml/min using helium as carrier gas. Initial oven temperature was held at 60°C for 1 min, increased to 90°C in 1 min, and then to 290°C in 25 min.
RESULTS AND DISCUSSION

Pyridylation of N-glycans of the major glycoproteins of PEW—More than 90% of the total carbohydrate was released from each PEW glycoprotein by the sequential digestion of PEW with trypsin/chymotrypsin followed by glycoamidase A, as determined by the orcinol-H$_2$SO$_4$ reaction (20) (data not shown). For mass spectrometric analysis (Fig. S1 of the Supplemental Material), a part of the N-glycan mixture was desialylated before PA-derivatization.

Mass spectrometric analyses of N-glycans—To rapidly evaluate the glycosylation difference among the four PEW glycoproteins, a total mass spectrometry profile was obtained for each of the permethyl derivatives of the desialylated PA-oligosaccharide pools (Fig. S1). From the molecular ion signals detected, as well as the common fragment ions afforded at $m/z$ 260 (HexNAc$^+$), 464 (Hex-HexNAc$^+$), and 668 (Hex-Hex-HexNAc$^+$) in low mass range (data not shown), it could be concluded that all PEW glycans are similarly heterogenous, differ mainly in the relative ratio of each of the complex type N-glycans identified (Table I). The absence of prominent fragment ions between $m/z$ 1000 and 2000 (data not shown) suggests that the trimannosyl core is mainly extended by ±Gal-(Gal-GlcNAc) antenna without significant amount of LacNAc repeats. This was further supported by the linkage analysis data which showed that all four PEW glycan pools gave similar partially methylated alditol acetates (PMAA) peaks but differ in relative amounts (Fig. S2 of the Supplemental Material). Terminal Gal, 4-linked Gal, and 4-linked GlcNAc were the three most prominent peaks detected, followed by 2,4-linked Man and terminal GlcNAc. These most prominent peaks corroborated the assignment of Gal-4Gal-4GlcNAc and Gal-4GlcNAc as the predominant termini. The identification of 2,4-linked Man indicated that
most species were at least triantennary, whereby the upper 6-arm could be non-branched (2-linked Man), 2,6-branched or 2,4,6-branched. In support of this, POM showed the highest amount of 2,4,6-linked Man. Conversely, 2-linked Man was most abundant in POT and low in POM and POA(hi). It was also relatively more abundant in POA(lo) than POA(hi). This is consistent with the distribution of the molecular ion profiles observed (Fig. S1). In addition, the relative ratio of 3,4,6-linked Man : 3,6-linked Man was the highest in POT, suggesting that POT has the highest proportion of bisecting GlcNAc which was nevertheless present in all PEW glycans. No 2-Gal, 3-Gal, and 6-Gal were detected from the desialylated samples of PEW-glycoproteins, suggesting that the linkage of Gal-Gal is only Gal1-4Gal.

In summary, FAB-Mass spectrometry and GC-EI-Mass spectrometry analysis of the permethylated derivatives of the desialylated PA-tagged N-glycans from PEW, yielded the following conclusions. 1) Gal(1,4)Gal(1,4)GlcNAc is the predominant terminal structure, and there were no Gal(1,2)Gal, Gal(1,3)Gal, or Gal(1,6)Gal linkages. This is in total agreement with the results independently obtained in the preceding paper (5)[Suzuki, N., et al, M1:01379]. 2) The trimannosyl core was mainly extended by ±Gal-(Gal-GlcNAc) antenna without significant amount of LacNAc repeats. 3) Extensive heterogeneity is largely a consequence of different permutation of the number of antennae, absence or presence of bisecting GlcNAc, and different location and degree of sialylation. Gal-GlcNAc termini could be due to incomplete capping with either sialylation or α-galactosylation, or, in the case of the desialylated samples, removal of the terminal sialic acids (Fig. S1). It is obvious that detailed characterization of all glycan chains present is not possible without great effort. We therefore chose to employ a facile 2D-HPLC mapping method to secure a comparative glycosylation profiles of the four PEW and homing in only on those more abundant and/or
characteristic peaks of common occurrence thus purified. The total mass spectrometry data profile was used as a starting reference point to interpret the HPLC mapping data and, in general, agrees well with the final conclusion drawn, apart from a few peaks which were not further identified and characterized by subsequent HPLC mapping (see Table I).

Separation of N-glycans by three successive HPLC steps—Separation of PA-oligosaccharides by DEAE-5PW column (first HPLC) yielded neutral, mono-sialyl, and di-sialyl oligosaccharide fractions (for example, Fig. S3 for the N-glycans from POT). All ionic charges in the PEW are attributable to α-sialic acids, since all acidic fractions changed to neutral species after Arthrobacter α-sialidase digestion.

The PA-glycan fractions separated on DEAE-5PW column were applied to the ODS column, using exactly the same chromatographic conditions for neutral (N), mono-sialyl (MS), and di-sialyl (DS) N-glycans from the POT, POM, and POAs (Fig.1). Molar ratios (calculated from their peak areas) of the N, MS, and DS fractions were, respectively, 40.8, 52.7, and 6.4% for POT; 25.4, 55.1, and 19.5% for POM; 36.5, 49.8, and 13.7% for POA(hi); and 28.4, 61.3, and 10.3% for POA(lo). As shown in Fig. 1, the N-glycan profiles released from the four different PEW glycoproteins were qualitatively similar but not quantitatively identical.

Each PA-labelled fraction from the ODS column was further fractionated by the molecular size using the amide-silica column. On the amide-silica column, the neutral fraction N-IX was separated into N-IXa and -IXb, and the N-X, into N-Xa and -Xb. MS-I was separated into MS-Ia and -Ib; MS-III, into MS-IIIa and -IIIb; and MS-V, into MS-Va, -Vb, and -Vc. Fig. S4 of the Supplemental Material shows an example of separation profiles.
of the neutral fraction N-IX and mono-sialyl fraction MS-V from POT. Other ODS fractions appeared as single peaks on amide-silica column.

**Elucidation of N-glycan structures obtained from PEW glycoproteins**—Since the branches of the predominant N-glycans from PEW terminate in the sequence of Galβ(1,4)Galβ(1,4), and this sequence was reported only once previously to be present in N-glycan (2), most, if not all, of the N-glycans in the PEW glycoproteins must have novel or unusual structures. Indeed, a total of 25 N-glycans were isolated and identified unambiguously (Table III) or tentatively (Table IV). The coordinate sets of these compounds do not match, by direct comparison, with any of the reference compounds available on the 2-D map accumulated prior to our studies (data not shown).

**Structures of N-I, N-III, N-VI, and N-IXb**—As shown in Fig. 2, after α–galactosidase digestion, N-I and N-VI changed into the known tetraantennary structures, 400.16 and 401.16, respectively. The number of α–galactose residues released were calculated from their GU (amide) before and after α–galactosidase digestion (12.9 vs. 9.8 and 13.0 vs. 9.9). On the average, GU (amide) of ca. 0.8 corresponds to one galactose residue (12). Consequently, we could assign 4 α-galactose residues being attached to the 4 β-galactoses in 400.16 and 401.16, respectively. The structures of N-I and N-VI were thus established (Table III). Assignment of singly and/or doubly charged molecular ions afforded by ESI-Mass spectrometry analysis of the permethylated PA-glycans (ESI-Mass spectrometry) further confirmed this. The measured molecular mass of permethylated N-I changed from 3855.8 (Hex₁₁HexNAc₆-PA) to 3039.6 (Hex₇HexNAc₆-PA) after α–galactosidase digestion. The difference of 816.2 corresponds to 4 mol of galactose residues. Likewise, the measured molecular mass of permethylated N-VI changed from 4101.2 (Hex₁₁HexNAc₇-PA) to 3284.7 (Hex₇HexNAc₇-
PA) upon α-glactosidase digestion, indicating the difference of 816.9 corresponds to 4 mol of galactose residues. Similarly, after α-glactosidase digestion, N-III and N-IXb released 3 mols of galactose residues, accompanied by the changes in GU(amide), 2.5 and 2.4, to become the known triantennary reference compounds, 300.8, and 301.8, respectively. In this case also, we could unambiguously assign 3 α-galactose residues to be on the 3 β-galactoses on the respective reference compounds, and the structures of N-III and N-IXb were thus elucidated. The molecular mass of permethylated N-IXb was 3447.9 (Hex$_9$HexNAc$_6$-PA) as determined by mass spectrometry analysis.

Structure of MS-Ib, MS-IIIa, MS-IIIb, and MS-Vc—Deciphering the 6- vs. 3-sialylation was by the differential changes of GU(amide) as reported previously (11,21). Briefly, after sialidase digestion, the GU(amide) of the sample increases by about 0.4 per mol of NeuAcα(2,3), but little change was observed for NeuAcα(2,6). In the $N$-glycans from pigeon egg white glycoproteins, all the Neu5Ac linkage seemed to be α(2-6)-linked. After α-galactosidase digestion, MS-Ib and MS-IIIb each released 3 mols of α-galactose residues and changed into the known reference tetraantennary compounds having a NeuAcα(2,6) attached to the Galβ(1,4)GlcNAc β(1,2)Manα(1,3) branch, 1A4-400.16 (10.0, 9.9) and 1A4-401.16 (13.1, 10.0), respectively. These results established the position of sialylation in these compounds. α-Galactosidase released 2 mols of α-galactose residues from MS-IIIa and MS-Vc and changed them into the known reference monosialyl triantennary compounds 1A1-300.8 (11.9, 8.2) and 1A1-301.8 (15.3, 8.3), respectively. Thus, the sialic acid residue was confined to be on the Galβ(1,4)GlcNAc β(1,2)Man α(1,3) branch in both cases, and both α-galactose residues could be assigned unambiguously to the two β-galactoses on the remaining branches. Mass spectrometry analyses of the permethyl derivatives revealed a
molecular mass of 3359.0 (NeuAc₁Hex₈HexNAc₆-PA) for MS-IIIa and 3605.2
(NeuAc₁Hex₈HexNAc₅-PA) for MS-Vc, in total agreement with the results from the 2-D
mapping. The structures of MS-IIIa and MS-Vc were thus solidly established (Table III).

Thus, the structures of 8 out of the 25 isolated oligosaccharides were directly
transformed to known reference structures. Thereafter, we were able to use these 8 structures
as new reference compounds for elucidation of the other compounds.

*Structures of N-II, N-V, N-Xb, and N-XI—α-Galactosidase released 3 mols of
galactoses from N-II and N-Xb and changed into known reference compounds, 400.16
(tetraantennary) and 401.16 (a tetraantennary structure with the bisecting GlcNAc),
respectively. Similarly, N-V and N-XI released 2 mols of galactose by α-galactosidase and
changed into known reference compounds, 300.8 (triantennary) and 301.8 (triantennary with
a bisecting-GlcNAc), respectively. To determine the position of the terminal β-galactosyl
residue in each of N-II, N-Xb, N-V, and N-XI, they were digested with β-galactosidase.
Upon such digestion, the GU(ODS) decreased greatly (0.8, 1.2, 1.2, and 1.3, respectively).
This can occur only when the galactose digested is at the position 28 (Fig. 3) among all four
possibilities. These results suggest the branch with terminal β-Gal is Gaβ(1,4)GlcNAc
β(1,2)Man α(1,3). As expected, each of the newly acquired reference compounds, MS-Ib,
MS-IIIb, MS-IIIa and MS-Vc (see discussions above), released a sialic acid residue by α-
sialidase digestion to become its neutral compounds, which were completely identical with
those of N-II, N-Xb, N-V, and N-XI, respectively (Fig. 4). The changes in GU(ODS) after
desialylation of MS-IIIb and MS-Vc are much larger (4.5 and 5.0, respectively) than those
shown in Fig. 3. Since the positions of sialylation in each of these compounds have been
established, the extraordinarily large ΔGU(ODS) upon desialylation can be interpreted as
caused by the bisecting GlcNAc. The data in Fig. 3 related to Nos. 31, 35, 40, and 46 are based on only a limited number of bisecting GlcNAc-containing N-glycans. When more data become available, we would be able to rationalize this phenomenon more satisfactorily. Since this observation can be useful for identification of bisecting GlcNAc, we confirmed the results by ESI-Mass spectrometry analysis of the permethyl derivatives of the relevant peaks. N-II, with a molecular mass determined by ESI-Mass spectrometry as 3652.2 (Hex$_{10}$HexNAc$_{6}$-PA) is tetraantennary and has 3 $\alpha$-galactosides, and it cannot have the bisecting GlcNAc. By contrast, N-Xb, having the same tetraantennary structure, gave a molecular mass of 3897.6 (Hex$_{10}$HexNAc$_{7}$-PA), and the extra GlcNAc can only be interpreted as bisecting GlcNAc. The observed difference in the molecular masses measured is also paralleled in the case of N-V (2997.9, Hex$_8$HexNAc$_5$-PA) and N-XI, (3242.7, Hex$_8$HexNAc$_6$-PA).

*Structures of MS-Ia, MS-IV and N-VII*—After $\alpha$-galactosidase digestion, MS-Ia and MS-IV released 2 mols of galactose and changed into known tetraantennary compounds, 1A4-400.16 and 1A4-401.16, respectively. Similarly, N-VII released 3 mols of galactose to become a known tetraantennary 401.16. To determine which one of these branches in these compounds are terminated with $\beta$-galactoside, MS-Ia, MS-IV and N-VII were sequentially digested with $\beta$-galactosidase and $\beta$-$N$-acetylhexosaminidase to transform them to $\alpha$-galactose-containing triantennary glycans (Fig. 5), which are indistinguishable from MS-IIIa ($\Delta$GU(ODS) = + 1.9), MS-Vc ($\Delta$GU(ODS) = + 2.4), and N-IXb ($\Delta$GU(ODS) = + 1.5), respectively. The structure of MS-IIIa, MS-Vc, and N-IXb have been determined by both 2-D mapping and mass spectrometry as described above. Furthermore the UC values in Fig. 3 show that after sequential digestion with $\beta$-galactosidase and $\beta$-$N$-acetylglucosaminidase,
only the branch including the positions 17 (0.2, ODS) and 9 (-2.1, ODS) gives large increase in ΔGU(ODS), i.e., -(0.2 - 2.1) = +1.9. It was clear that the branch without α-galactose is the branch including the positions 17 and 9.

**Structure of DS-I, a disialyl oligosaccharide**—DS-I was the predominant oligosaccharide in di-sialyl N-glycans in all four PEW glycoproteins (Fig. 1). After α-galactosidase and sialidase digestion, DS-I was transformed into a known tetraantennary glycan, 400.16 (Fig. 6). The change of the GU(amide) after these digestions indicates that DS-I contains 2 α-galactoses along with 2 sialic acid residues at the nonreducing termini of 400.16. The change of the GU(amide) was 1.6 (11.4 minus 9.8), which can be rationalized as loss of 2 NeuAc and 2 α-galactose. To determine the exact position of NeuAc on these four residues, DS-I was first digested sequentially with α-galactosidase and β-galactosidase. After denaturing the enzymes by 10-min boiling, the reaction product was further digested with α-sialidase, to yield a product totally identical with 400.6 (Fig. 6) having the coordinates of (9.9, 7.9). Therefore, disialylation must be on the branches, Galβ4GlcNAcβ2Manα3 and Galβ4GlcNAcβ2Manα6 in 400.6.

**Structures of other N-glycans**—Structures of other N-glycans were more difficult to determine, mainly because of the limited amounts available (Table IV).

i) **MS-II and N-IV**—Sialidase digestion of MS-II, transformed it to a product indistinguishable from N-IV, indicating that the structure of N-IV is shared in MS-II. This was confirmed by ESI-Mass spectrometry analysis where the measured molecular masses for permethylated MS-II (NeuAc1Hex12HexNAc7-PA) and N-IV (Hex12HexNAc7-PA) were 4667.6 and 4305.2 respectively.
Digestion of MS-II with \(\alpha\)-galactosidase changed GU(amide) from 14.2 to 11.0, corresponding to the loss of 4 galactose residues. With a sialic acid and four galactoses at the terminals, MS-II is clearly pentaantennary. Similar digestion of N-IV with \(\alpha\)-galactosidase also resulted in the release of 4 mol of galactose, in agreement with the above conclusion. The absence of bisecting GlcNAc in MS-II was shown by exhaustive digestion with sialidase, \(\alpha\)- and \(\beta\)-galactosidase. The product was clearly different from the GlcNAc-terminated pentaantennary structure with bisecting GlcNAc, Code No. 501.1 (Table II). Since there are no reference compounds directly comparable with either MS-II or N-IV, we could only estimate their structures by the following deduction. The increase in GU(ODS) upon desialylation of MS-II (11.2 - 9.7=1.5) is agreeable with the UC(ODS) at the position 46 (Fig. 3), and the structures of MS-II and N-IV shown in Table IV are most probable.

Unfortunately, our present database does not include the UC values at the positions labeled X and Y in Fig. 3 (for the NeuAc\(\alpha\)2,6 and Gal\(\beta\)1,4 residues extended from the position 10) to allow direct comparison to exclude these possibilities absolutely.

Prior to our studies, the only pentaantennary structures reported were in chicken ovomucoid (8,22) and turtle dove ovomucoid (2). The former contains the bisecting GlcNAc, and the latter is different from any of the pentaantennary structures we have found in the major PEW glycoproteins with respect to the position of sialic acid. In the structure proposed for turtle dove ovomucoid, the sialic acid was placed on the branch of Gal\(\beta\)4GlcNAc\(\beta\)4Man\(\alpha\)3, whereas the closest structure among the PEW N-glycans are MS-II, in which sialic acid is on the Gal\(\beta\)4GlcNAc\(\beta\)2Man\(\alpha\)3 branch (see below).

\(\text{ii) Structure of N-VIII—After }\alpha\text{-galactosidase digestion, the coordinates of N-VIII (13.3, 11.5) changed to (18.7, 9.0), suggesting the release of 3 galactoses (}\Delta\text{GU(amide)} = \)
2.5). The coordinates of the product correspond to a tetraantennary glycan, 401.16 (GU 18.6 and 9.9) which is devoid of the branch containing the X, Y positions) minus one galactose residue (albeit of unknown location). The de-α-galactosylation product, however, could not be matched on the ODS column with the respective de-β-galactosylation products from N-VII nor N-Xb (see Table III). Therefore, the missing β-galactoside cannot be on the positions 17 or 28, which leaves the positions 21 or 24 as the only possibilities (Fig. 3). The α-galactosidase digestion product showed GU(18.7, 9.0), which differ from those of 401.16 (18.6, 10.0) by +0.1 and -1.0, suggesting that the original β-galactose to be at the position 21.

iii) Structures of N-IXa and N-Xa—Digestion of N-Xa with α-galactosidase resulted in loss of two galactosyl residues, and the product was indistinguishable from a known reference, 301.8 (a triantennary with bisecting GlcNAc. See Table II). This determines the pattern of the triantennary branching for N-Xa. β-Galactosidase digestion caused the coordinates of N-Xa (15.1, 9.7) to coincide with those of N-IXa (14.7, 9.1), thus N-IXa has the same triantennary structure as N-Xa. Furthermore from the values in Fig. 3, the position of the β-galactosyl terminus in N-Xa is most likely to be the positions 24 having UC values of (0.6, 0.6) (Fig. 3), because the GU of the missing β-galactose residue from N-IXa is 0.4 (i.e., 15.1 - 14.7) and 0.6 (i.e., 9.7 - 9.1). Therefore, the structures of N-IXa, N-Xa perhaps are as shown in Table IV.

iv) Structure of MS-VI, MS-Va, and MS-Vb—After α-galactosidase digestion, MS-VI (14.8, 9.1) released one mole of α-galactose and changed into a known mono-sialyl triantennary compound, 1A1-301.8 (15.3, 8.3) as shown in Table II. Thus, the sialic acid residue was confirmed to be at the position 46. After β-galactosidase digestion, the coordinates of MS-VI (14.8, 9.1), became totally coincidental with those of MS-Vb (13.9,
indicating that MS-VI and MS-Vb are related to each other. Thus MS-VI, and consequently MS-Vb also, are triantennary with the bisecting GlcNAc. The magnitudes of the GU changes upon digestion of MS-VI with β-galactosidase, 0.9 on ODS and 0.6 on amide, indicate the non-reducing β-galactosylation in MS-VI is more likely to be at the position No. 24 than at No. 21 (Fig. 3).

Upon de-α-galactosylation, the GU(amide) of MS-Va (8.0) decreased by 0.7, indicating the release of one α-galactose residue. Upon successive digestion with α-galactosidase and α-sialidase, the products from MS-Va and MS-Vb showed coordinates of (19.1, 7.2) and (18.9, 7.5), respectively. The magnitude of ΔGU(ODS and amide) values between the products from MS-Va and MS-Vb are +0.2 (i.e., 19.1 - 18.9), and -0.3 (i.e., 7.2 - 7.5). From Fig. 3, the difference in ΔGU between the positions 24 and 21 are (+0.4, -0.3). Since MS-Vb, distinctively different from MS-Va, has the position 21, the position 24 can fit reasonably to MS-Va.

t) Structure of DS-II—Digestion of DS-II (16.3, 9.1) with sialidase and α-galactosidase resulted in a product totally identical to 301.8, triantennary with bisecting GlcNAc (Table II). However, the positions of the α-galactose and NeuAc residues could not be unambiguously assigned.

Distribution of various N-glycans—Fig. 7 shows a distribution profile of N-glycans in PEW glycoproteins. Each N-glycan contained one to four Galα(1,4)Galβ(1,4) sequences at the nonreducing termini. All N-acetylneuraminic acids were α(2,6)-linked, and they are located on the Galβ4GlcNAcβ2Manα3 branch in all monosialyl and DS-I structures. In POT, triantennary structures with bisecting GlcNAc (N-IXb and MS-Vc) were predominant. By contrast, in the other three glycoproteins, tetraantennary (with and without bisecting GlcNAc)
structures were predominant. They are N-II and MS-Ib in POM, N-VI and MS-Ib in POA(hi), and N-I and MS-Ib in POA(lo). Pentaantenary structures were found mostly in POA(hi) and POM as mono-sialyl and without bisecting GlcNAc type.

Conclusion

The detailed structural studies of the major N-glycans presented here clearly indicate the rich galabiose content is the reason why the PEW glycoproteins serve as inhibitors for uropathogenic E.coli (23-25). Every oligosaccharide examined contain at least one and up to 4 galabiose sequence. Thus, pigeon egg white may be an inexpensive natural resource for the galabiose containing N-glycans. Which other species contain galabiose element in their N-glycans is an interesting question to gain insight into the evolutionary significance of galabiose formation in glycoproteins.

Although monosialyl oligosaccharides are the predominant group in each of the glycoproteins, the sialylation seems confined to one of the branches only, and this branch is also sialylated in the disialyl species. Together with the fact that α-galactosyl terminals have no sialylation, this unique sialylation pattern may have important significance.
Acknowledgment

This work was supported by National Institutes of Health Research Grant DKO9970 (NS, YCL), by Academia Sinica (KHK), Mitsukan Co. Ltd. (NT), and National Institutes of Health grants DK-47504 (JRJ). The assistance of Mr. Pei-Lun Tsai in the ESI-Mass spectrometry analysis is greatly appreciated. The authors are also grateful for Dr. Noboru Tomiya for his careful reading of the manuscript and active discussion on the interpretation of the HPLC data.
References


Footnotes

The on-line version of this article (available at http://www.jbc.org) contains Supplemental Material including profiles on mass spectrometric analyses and HPLC.

1. Abbreviations used are: FAB, fast atom/ion bombardment; GC-EI, gas chromatography-electron impact; GU(amide), glucose unit value on the amide column; GU(ODS), glucose unit value on the ODS column; Hex, hexose; HexNAc, N-acetylhexosamine; HPLC, high-performance liquid chromatography; NeuAc, N-acetylneuraminic acid; ODS, octadecylsilica; PA, pyridylamine; PEW, pigeon egg white; PMAA, partially methylated alditol acetates; POA, pigeon ovalbumin; POM, pigeon ovomucoid; POT, pigeon ovotransferrin; TFA, trifluoroacetic acid; TOF, time of flight. UC, the contribution of the individual monosaccharide unit in a specific position expressed in glucose unit (12).

2. For all the structures of the PA-oligosaccharides and their code numbers so far accumulated, please refer to web site, http://www.gak.co.jp/FCCA. Whenever coordinates are cited in this paper, they are always listed in the order of GU(ODS), GU(amide).
FIGURE LEGENDS

Fig. 1. Comparison on ODS column, of the profiles of pyridylamino N-glycans obtained from four different PEW glycoproteins. Each of the neutral, mono, and disialyl N-glycans from POT (T), POM (M), POA(hi) (Ah), and POA(lo) (Al) was separated on the first DEAE column, and then separated on the second ODS column. The profiles of neutral and disialyl are enlarged by a multiplier of 2.

Fig. 2. Structural analyses of eight N-glycans (N-I, N-III, N-VI, N-IXb, MS-Ib, MS-IIIa, MS-IIIb, and MS-Vc) by a combination of digestion with α-galactosidase and 2-D mapping technique. A portion (about 50 pmol each) of the eight PA-glycans was digested with α-galactosidase. The elution times on ODS and amide-silica columns of the eight N-glycans and their α-galactosidase digests are plotted on a 2-D sugar map (expressed as glucose unit) with reference compounds 400.16, 401.16, 1A4-400.16, 1A4-401.16, 300.8, 301.8, 1A1-300.8, and 1A1-301.8. Arrows indicate the direction of changes of the coordinates of N-glycans after digestion with α-galactosidase. Structures of reference compounds are shown in Table II.

Fig. 3. Diagram of the partial UC values (12) relevant to this manuscript. On the left-hand side of each box are the component numbers, and the right hand side box shows the sugar residue on top, UC(ODS) at the middle, and UC(amide) at the bottom. From the total 54 component numbers, only those relevant to the discussions in this manuscript were selected.

Fig. 4. Relationship between neutral compounds (N-II, N-Xb, N-V and N-XI) and newly obtained reference compounds (MS-Ib, MS-IIIb, MS-IIIa and MS-Vc). By α-sialidase digestion, MS-Ib, MS-IIIb, MS-IIIa and MS-Vc each released one sialic acid
(denoted as –S), and became a neutral compound confirmed to be identical with that of
N-II, N-Xb, N-V and N-XI, respectively. Arrows indicate the directions of changes of
the coordinates of N-glycans after digestion with α-sialidase.

\[
\begin{align*}
\text{Gα4Gβ4GNβ6} & \quad \rightarrow \quad \text{Gα4Gβ4GNβ6} \\
\text{Gα4Gβ4GNβ2} & \quad \rightarrow \quad \text{Gα4Gβ4GNβ2} \\
\text{Gα4Gβ4GNβ4} & \quad \rightarrow \quad \text{Gα4Gβ4GNβ4} \\
\text{MS-IIIb} & \quad \rightarrow \quad \text{N-Xb} \\
\text{Gα4Gβ4GNβ2} & \quad \rightarrow \quad \text{Gα4Gβ4GNβ2} \\
\text{Gα4Gβ4GNβ4} & \quad \rightarrow \quad \text{Gα4Gβ4GNβ4} \\
\text{MS-IIIa} & \quad \rightarrow \quad \text{N-V} \\
\text{MS-Vc} & \quad \rightarrow \quad \text{N-XI}
\end{align*}
\]
Fig. 5. Structural analyses of N-glycans MS-Ia, MS-IV, and N-VII by a combination of sequential digestion with β-galactosidase and β-N-acetylhexosaminidase and 2-D mapping technique. About 50 pmol each of N-glycan (MS-Ia, MS-IV, and N-VII) was digested sequentially with β-galactosidase and β-N-acetylhexosaminidase. The values of GU(ODS, amide) of the three N-glycans and the products from exoglycosidase digests were plotted with reference compounds MS-IIIa, MS-Vc and N-IXb. Arrows indicate the directions of changes of the GU after digestion with β-galactosidase and β-N-acetylhexosaminidase.

\[
\begin{align*}
\text{MS-Ia} & : \quad \text{Gβ4GN\(\beta\)6} \\
\text{MS-IV} & : \quad \text{Gα4Gβ4GN\(\beta\)2} \\
\text{N-VII} & : \quad \text{Gα4Gβ4GN\(\beta\)2}
\end{align*}
\]
**Fig. 6. Diagram of structural analysis of DS-I.** DS-I was transformed into 400.6 and 400.16 by enzymatic digestion indicated.

**Fig. 7. Comparison of distribution profiles of N-glycans in PEW glycoproteins.**

Characteristic N-glycan distribution was expressed on the basis of individual glycoproteins. The suffixes, -A and -N indicate “acidic” and “neutral” fractions.
<table>
<thead>
<tr>
<th>[M+H]^+</th>
<th>Assignment, based on a common trimannosyl chitobiose core¹</th>
<th>Correspondence to structures in Tables III and IV²</th>
</tr>
</thead>
<tbody>
<tr>
<td>2386</td>
<td>Core + Hex₂HexNAc₃</td>
<td>MS-Va -SA³, MS-Vb -SA</td>
</tr>
<tr>
<td>2427</td>
<td>Core + Hex₁HexNAc₄</td>
<td>N-V, MS-IIIa -SA</td>
</tr>
<tr>
<td>2590</td>
<td>Core + Hex₃HexNAc₃</td>
<td>N-IXa, MS-VI -SA, DS-II -2SA</td>
</tr>
<tr>
<td>2631</td>
<td>Core + Hex₅HexNAc₄</td>
<td>N-III</td>
</tr>
<tr>
<td>2794</td>
<td>Core + Hex₄HexNAc₅</td>
<td>N-XI, N-Xa, MS-Vc -SA</td>
</tr>
<tr>
<td>2835</td>
<td>Core + Hex₃HexNAc₄</td>
<td>N-IXb, MS-Ia -SA, DS-I -2SA</td>
</tr>
<tr>
<td>2998</td>
<td>Core + Hex₅HexNAc₃</td>
<td>N-II, MS-Ib -SA</td>
</tr>
<tr>
<td>3039</td>
<td>Core + Hex₄HexNAc₄</td>
<td>N-VIII, MS-IV -SA</td>
</tr>
<tr>
<td>3202</td>
<td>Core + Hex₆HexNAc₅</td>
<td>N-I</td>
</tr>
<tr>
<td>3243</td>
<td>Core + Hex₅HexNAc₅</td>
<td>N-VIII, N-Xb, MS-IIIb -SA</td>
</tr>
<tr>
<td>3284</td>
<td>Core + Hex₇HexNAc₅</td>
<td>N-VI</td>
</tr>
<tr>
<td>3447</td>
<td>Core + Hex₅HexNAc₅</td>
<td>N-IV, MS-IV -SA</td>
</tr>
<tr>
<td>3488</td>
<td>Core + Hex₆HexNAc₅</td>
<td>N-IV, MS-IV -SA</td>
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<td>3651</td>
<td>Core + Hex₇HexNAc₅</td>
<td>N-IV, MS-IV -SA</td>
</tr>
<tr>
<td>3692</td>
<td>Core + Hex₆HexNAc₅</td>
<td>N-IV, MS-IV -SA</td>
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<td>3856</td>
<td>Core + Hex₇HexNAc₅</td>
<td>N-IV, MS-IV -SA</td>
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<tr>
<td>3989</td>
<td>Core + Hex₈HexNAc₅</td>
<td>N-IV, MS-IV -SA</td>
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<td>4101</td>
<td>Core + Hex₈HexNAc₅</td>
<td>N-IV, MS-IV -SA</td>
</tr>
<tr>
<td>4306</td>
<td>Core + Hex₉HexNAc₅</td>
<td>N-IV, MS-IV -SA</td>
</tr>
</tbody>
</table>

¹. All components listed could be detected in each of the PEW PA-oligosaccharide pools but differed in relative amounts. Most could be further assigned as tri-, tetra- or pentaantennary structures based on the number of GlcNAc residues contained. However, the presence of bisecting GlcNAc should also be taken into consideration. The maximum number of GlcNAc residue in addition to the two of the chitobiose core is 5, which suggests that a pentaantennary structure in general does not also carry a bisecting GlcNAc.

². Not all PA-oligosaccharides could be identified by the 2-D map method, probably due to the limited quantities.

³. "-SA" denotes loss of NeuAc.
**TABLE II** Structures of PA-glycans used as reference compounds. 
*R* denotes *Manβ4GlcNAcβ4GlcNAc*

<table>
<thead>
<tr>
<th>Peak</th>
<th>Structure of N-glycans</th>
<th>Coordinates</th>
</tr>
</thead>
<tbody>
<tr>
<td>300.8</td>
<td>Manα6 ( \rightarrow ) Galβ4GlcNAcβ2, R ( \rightarrow ) Galβ4GlcNAc4</td>
<td>12.7, 8.3</td>
</tr>
<tr>
<td></td>
<td>Manα3 ( \rightarrow ) Galβ4GlcNAcβ2 ( \rightarrow ) Manα3 ( \rightarrow ) Galβ4GlcNAcβ2</td>
<td></td>
</tr>
<tr>
<td>301.8</td>
<td>Manα6 ( \rightarrow ) Galβ4GlcNAcβ2, GlcNAcβ4R ( \rightarrow ) Galβ4GlcNAcβ2</td>
<td>20.2, 8.2</td>
</tr>
<tr>
<td></td>
<td>Manα3 ( \rightarrow ) Galβ4GlcNAcβ2 ( \rightarrow ) Manα3 ( \rightarrow ) Galβ4GlcNAcβ2</td>
<td></td>
</tr>
<tr>
<td>1A1-300.8</td>
<td>Manα6 ( \rightarrow ) Galβ4GlcNAcβ2, R ( \rightarrow ) Galβ4GlcNAcβ4 ( \rightarrow ) Manα3 ( \rightarrow ) Neu5Acα6Galβ4GlcNAcβ2</td>
<td>11.9, 8.2</td>
</tr>
</tbody>
</table>
3A3-300.8
Neu5Acα3Galβ4GlcNAcβ2
Manα3
R
7.6
15.3
3A3-300.8
Neu5Acα3Galβ4GlcNAcβ4
Manα3
Neu5Acα6Galβ4GlcNAcβ2
Manα6
Galβ4GlcNAcβ2
GlcNAcβ4R
8.3
1A1-301.8
Galβ4GlcNAcβ4
Manα3
Neu5Acα6Galβ4GlcNAcβ2
GlcNAcβ6
Manα6
Galβ4GlcNAcβ2
R
7.9
400.6
GlcNAcβ4
Manα3
Galβ4GlcNAcβ2
Galβ4GlcNAcβ6
Manα6
Galβ4GlcNAcβ2
R
10.2
400.16
Galβ4GlcNAcβ4
Manα3
Galβ4GlcNAcβ2
R
9.8
TABLE III

PA-glycans obtained from POT, POM, POA(hi) and POA (lo) with unambiguous structures.

R denotes Man\(4\)GlcNAc\(4\)GlcNAc

<table>
<thead>
<tr>
<th>Peak</th>
<th>Structure of N-glycans</th>
<th>Coordinates</th>
<th>Relative quantity (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Code</td>
<td>ODS Amide</td>
<td>POT</td>
<td>POM</td>
</tr>
<tr>
<td>N-I</td>
<td>Gal(\alpha)Gal(\beta)GlcNAc(\beta)</td>
<td>8.5</td>
<td>4.2</td>
</tr>
<tr>
<td></td>
<td>Man(\alpha)6</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>Gal(\alpha)Gal(\beta)GlcNAc(\beta)2</td>
<td>R</td>
<td>12.9</td>
</tr>
<tr>
<td></td>
<td>Gal(\alpha)Gal(\beta)GlcNAc(\beta)4</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>Man(\alpha)3</td>
<td></td>
<td></td>
</tr>
<tr>
<td>N-II</td>
<td>Gal(\alpha)Gal(\beta)GlcNAc(\beta)</td>
<td>9.8</td>
<td>2.2</td>
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<tr>
<td></td>
<td>Man(\alpha)6</td>
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<td></td>
</tr>
<tr>
<td></td>
<td>Gal(\alpha)Gal(\beta)GlcNAc(\beta)2</td>
<td>R</td>
<td>12.3</td>
</tr>
<tr>
<td></td>
<td>Gal(\alpha)Gal(\beta)GlcNAc(\beta)4</td>
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<tr>
<td></td>
<td>Man(\alpha)3</td>
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<td></td>
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<tr>
<td>N-III</td>
<td>Gal(\alpha)Gal(\beta)GlcNAc(\beta)2Man(\alpha)6</td>
<td>10.4</td>
<td>4.4</td>
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<tr>
<td></td>
<td>Chain Structure</td>
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<tr>
<td><strong>N-V</strong></td>
<td>Galβ4Gaβ4GlcNAcβ2Manα6</td>
<td>11.6</td>
<td>-</td>
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<tr>
<td></td>
<td>Galβ4Gaβ4GlcNAcβ4</td>
<td>R</td>
<td>9.7</td>
</tr>
<tr>
<td></td>
<td>Galβ4GlcNAcβ2</td>
<td>Manα3</td>
<td></td>
</tr>
<tr>
<td><strong>N-VI</strong></td>
<td>Galβ4Gaβ4GlcNAcβ6</td>
<td>Manα6</td>
<td>12.2</td>
</tr>
<tr>
<td></td>
<td>Galβ4Gaβ4GlcNAcβ2</td>
<td>GlcNAcβ4R</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Galβ4Gaβ4GlcNAcβ4</td>
<td>Manα3</td>
<td></td>
</tr>
<tr>
<td><strong>N-VII</strong></td>
<td>Galβ4Gaβ4GlcNAcβ2</td>
<td>Manα6</td>
<td>13.0</td>
</tr>
<tr>
<td></td>
<td>Galβ4Gaβ4GlcNAcβ2</td>
<td>GlcNAcβ4R</td>
<td>12.2</td>
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<td></td>
<td>Galβ4Gaβ4GlcNAcβ4</td>
<td>Manα3</td>
<td></td>
</tr>
<tr>
<td><strong>N-IXb</strong></td>
<td>Galβ4Gaβ4GlcNAcβ2Manα6</td>
<td>14.5</td>
<td>9.6</td>
</tr>
<tr>
<td></td>
<td>Galβ4Gaβ4GlcNAcβ2</td>
<td>GlcNAcβ4R</td>
<td>10.6</td>
</tr>
<tr>
<td></td>
<td>Galβ4Gaβ4GlcNAcβ4</td>
<td>Manα3</td>
<td></td>
</tr>
</tbody>
</table>
Galβ4Galβ4GlcNAcβ6
   \quad \text{Man}α6
Galβ4Galβ4GlcNAcβ2 \quad \text{GlcNAc}β4R \quad 15.4 \quad - \quad 1.8 \quad 1.9 \quad 1.1
Galβ4Galβ4GlcNAcβ4
   \quad \text{Man}α3
Galβ4GlcNAcβ2

N-Xb

Galβ4Galβ4GlcNAcβ2Manα6 \quad 19.0 \quad 4.3 \quad 0.4 \quad 1.5 \quad 2.6
\quad \text{GlcNAc}β4R \quad 9.7
Galβ4Galβ4GlcNAcβ4
   \quad \text{Man}α3
Galβ4GlcNAcβ2

Mono-sialyl

Galβ4GlcNAcβ6
   \quad \text{Man}α6
Galβ4Galβ4GlcNAcβ2 \quad 9.1 \quad - \quad 6.2 \quad - \quad 7.2
\quad \text{R}
Galβ4Galβ4GlcNAcβ4
   \quad \text{Man}α3
Neu5Acα6Galβ4GlcNAcβ2

MS-Ia

Galβ4Galβ4GlcNAcβ6
   \quad \text{Man}α6
Galβ4Galβ4GlcNAcβ2 \quad 9.1 \quad 12.7 \quad 14.8 \quad 20.9 \quad 17.1
\quad \text{R}
Galβ4Galβ4GlcNAcβ4
   \quad \text{Man}α3
Neu5Acα6Galβ4GlcNAcβ2

MS-Ib
MS-IIIa
\[
\begin{align*}
\text{Gal}^\beta_4\text{Ga}^\beta_4\text{GlcNAc}^{\beta_2}\text{Man}^{\alpha_6} \\
\text{Gal}^\beta_4\text{Ga}^\beta_4\text{GlcNAc}^{\beta_4} &\quad \text{R} \quad \text{Man}^{\alpha_3} \\
\text{Neu5Ac}^{\alpha_6}\text{Ga}^\beta_4\text{GlcNAc}^{\beta_2} \\
\text{Gal}^\beta_4\text{Ga}^\beta_4\text{GlcNAc}^{\beta_6} &\quad \text{Man}^{\alpha_6} \\
\text{GlcNAc}^{\beta_4}\text{R} \\
\end{align*}
\]

\[
\begin{array}{cccc}
11.0 & 10.2 & 4.7 & 1.5 \\
9.9 & 4.2 \\
\end{array}
\]

MS-IIIb
\[
\begin{align*}
\text{Gal}^\beta_4\text{Ga}^\beta_4\text{GlcNAc}^{\beta_2} \\
\text{GlcNAc}^{\beta_4}\text{R} \quad \text{Man}^{\alpha_3} \\
\text{Neu5Ac}^{\alpha_6}\text{Ga}^\beta_4\text{GlcNAc}^{\beta_2} \\
\text{Gal}^\beta_4\text{Ga}^\beta_4\text{GlcNAc}^{\beta_4} &\quad \text{Man}^{\alpha_3} \\
\end{align*}
\]

\[
\begin{array}{cccc}
10.9 & - & 5.3 & 6.7 \\
12.4 & 7.4 \\
\end{array}
\]

MS-IV
\[
\begin{align*}
\text{Gal}^\beta_4\text{Ga}^\beta_4\text{GlcNAc}^{\beta_2} \\
\text{GlcNAc}^{\beta_4}\text{R} \quad \text{Man}^{\alpha_3} \\
\text{Neu5Ac}^{\alpha_6}\text{Ga}^\beta_4\text{GlcNAc}^{\beta_2} \\
\text{Gal}^\beta_4\text{Ga}^\beta_4\text{GlcNAc}^{\beta_4} &\quad \text{Man}^{\alpha_3} \\
\end{align*}
\]

\[
\begin{array}{cccc}
11.6 & - & 7.6 & 3.5 \\
11.7 & 2.8 \\
\end{array}
\]

MS-Vc
\[
\begin{align*}
\text{Gal}^\beta_4\text{Ga}^\beta_4\text{GlcNAc}^{\beta_2}\text{Man}^{\alpha_6} \\
\text{GlcNAc}^{\beta_4}\text{R} \\
\text{Neu5Ac}^{\alpha_6}\text{Ga}^\beta_4\text{GlcNAc}^{\beta_2} \\
\text{Gal}^\beta_4\text{Ga}^\beta_4\text{GlcNAc}^{\beta_4} &\quad \text{Man}^{\alpha_3} \\
\end{align*}
\]

\[
\begin{array}{cccc}
14.0 & 13.9 & 6.0 & 5.6 \\
10.0 & 8.3 \\
\end{array}
\]

37
Di-sialyl

DS-I

\[
\text{Gal}\alpha_4\text{Ga} \beta_4\text{GlcNAc}\beta_6
\]

\[
\text{Man}\alpha_3
\]

\[
\text{Neu5Ac}\alpha_6\text{Ga} \beta_4\text{GlcNAc}\beta_2
\]

<table>
<thead>
<tr>
<th>R</th>
<th>8.2</th>
<th>4.9</th>
<th>18.9</th>
<th>13.7</th>
<th>10.2</th>
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<tr>
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<td>11.4</td>
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TABLE IV

Proposed structures of PA-glycans from POT, POM, POA(hi) and POA (lo) deduced via UC values. \( R \) denotes \( \text{Man}\beta_4\text{GlcNAc}\beta_4\text{GlcNAc} \)

<table>
<thead>
<tr>
<th>Peak</th>
<th>Structure of ( N )-glycans</th>
<th>Coordinates</th>
<th>Relative quantity (%)</th>
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<tr>
<td>Code</td>
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<td>ODS Amide</td>
<td>POT</td>
</tr>
<tr>
<td>Amide Neutral</td>
<td>( \text{GlcNAc}\beta_2 ) ( \text{Gal}\beta_4\text{GlcNAc}\beta_6 )</td>
<td>11.2</td>
<td>-</td>
</tr>
<tr>
<td>N-IV</td>
<td>( \text{GlcNAc}\beta_2 ) ( \text{Man}\alpha_6 ) ( \text{Gal}\beta_4\text{GlcNAc}\beta_4 )</td>
<td>R 14.1</td>
<td>-</td>
</tr>
<tr>
<td>N-VIII</td>
<td>( \text{GlcNAc}\beta_4 ) ( \text{GlcNAc}\beta_4 ) ( \text{Man}\alpha_3 )</td>
<td>13.3</td>
<td>1.6</td>
</tr>
<tr>
<td></td>
<td>( \text{Gal}\beta_4\text{GlcNAc}\beta_2 )</td>
<td>11.5</td>
<td>-</td>
</tr>
</tbody>
</table>
Gal\(\alpha4\)Gal\(\beta4\)GlcNAc\(\beta2\)Man\(\alpha6\)GlcNAc\(\beta4\)R
\hspace{1cm}Man\(\alpha3\)
Gal\(\alpha4\)Gal\(\beta4\)GlcNAc\(\beta2\)

Gal\(\alpha4\)Gal\(\beta4\)GlcNAc\(\beta2\)Man\(\alpha6\)
\hspace{1cm}GlcNAc\(\beta4\)R
\hspace{1cm}Gal\(\beta4\)GlcNAc\(\beta4\)
\hspace{1cm}Man\(\alpha3\)
Gal\(\alpha4\)Gal\(\beta4\)GlcNAc\(\beta2\)

Mono-sialyl
Gal\(\alpha4\)Gal\(\beta4\)GlcNAc\(\beta6\)
Gal\(\alpha4\)Gal\(\beta4\)GlcNAc\(\beta4\)-Man\(\alpha6\)
Gal\(\alpha4\)Gal\(\beta4\)GlcNAc\(\beta2\)
\hspace{1cm}R
\hspace{1cm}9.7
\hspace{1cm}3.4
\hspace{1cm}8.0
\hspace{1cm}9.4
\hspace{1cm}5.9
Gal\(\alpha4\)Gal\(\beta4\)GlcNAc\(\beta4\)
\hspace{1cm}Man\(\alpha3\)
Neu5Ac\(\alpha6\)Ga\(\beta4\)GlcNAc\(\beta2\)

MS-II
GlcNAc\(\beta2\)Man\(\alpha6\)
\hspace{1cm}GlcNAc\(\beta4\)R
\hspace{1cm}Gal\(\alpha4\)Gal\(\beta4\)GlcNAc\(\beta4\)
\hspace{1cm}Man\(\alpha3\)
Neu5Ac\(\alpha6\)Ga\(\beta4\)GlcNAc\(\beta2\)

MS-Va
GlcNAc\(\beta2\)Man\(\alpha6\)
\hspace{1cm}GlcNAc\(\beta4\)R
\hspace{1cm}Gal\(\alpha4\)Gal\(\beta4\)GlcNAc\(\beta4\)
\hspace{1cm}Man\(\alpha3\)
Neu5Ac\(\alpha6\)Ga\(\beta4\)GlcNAc\(\beta2\)

MS-Vb
Gal\(\alpha4\)Ga\(\beta4\)GlcNAc\(\beta2\)Man\(\alpha6\)
\hspace{1cm}GlcNAc\(\beta4\)R
\hspace{1cm}GlcNAc\(\beta4\)
\hspace{1cm}Man\(\alpha3\)
Neu5Ac\(\alpha6\)Ga\(\beta4\)GlcNAc\(\beta2\)
Gal\(\alpha\)4Gal\(\beta\)4GlcNAc\(\beta\)2Man\(\alpha\)6
\GlcNAc\(\beta\)4R
\Gal\(\beta\)4GlcNAc\(\beta\)4
\Man\(\alpha\)3
Neu5Ac\(\alpha\)6Gal\(\beta\)4GlcNAc\(\beta\)2

**Di-sialyl**

Neu5Ac\(\alpha\)6Gal\(\beta\)4GlcNAc\(\beta\)2Man\(\alpha\)6
\GlcNAc\(\beta\)4R
\Gal\(\alpha\)4Gal\(\beta\)4GlcNAc\(\beta\)4
\Man\(\alpha\)3
Neu5Ac\(\alpha\)6Gal\(\beta\)4GlcNAc\(\beta\)2

<table>
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<tr>
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<th></th>
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<th>DS-II</th>
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<tr>
<td></td>
<td>GlcNAc4R</td>
<td></td>
<td></td>
<td></td>
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<tr>
<td></td>
<td>Galβ4GlcNAcβ4</td>
<td></td>
<td></td>
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<tr>
<td></td>
<td>Manα3</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>Neu5Acα6Galβ4GlcNAcβ2</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>14.8</td>
<td>3.8</td>
<td>1.1</td>
<td>1.0</td>
<td>6.5</td>
<td></td>
</tr>
<tr>
<td>9.1</td>
<td>9.4</td>
<td>1.6</td>
<td>0.4</td>
<td>-</td>
<td></td>
</tr>
<tr>
<td>16.3</td>
<td>1.6</td>
<td>0.4</td>
<td>-</td>
<td>-</td>
<td></td>
</tr>
</tbody>
</table>
Figure 1 Takahashi et al.
Figure 4 Takahashi et al.
Figure 5 Takahashi et al.
Figure 6 Takahashi et al.

Galα4Galβ4GlcNAcβ6

Manα6

Neu5Acα6Galβ4GlcNAcβ2
Galα4Galβ4GlcNAcβ4

Manα3

Neu5Acα6Galβ4GlcNAcβ2

i) - αGal
ii) - βGal
iii) - SA

GlcNAcβ6

Manα6

Galβ4GlcNAcβ2

GlcNAcβ4

Manα3

Galβ4GlcNAcβ2

400.6 (9.9, 7.9)

R

DS-I (8.2, 11.4)

R

Galβ4GlcNAcβ2

400.16 (10.2, 9.8)

i) - αGal,
ii) - SA

Galβ4GlcNAcβ2

Manα6

Galβ4GlcNAcβ2

Manα3
Figure 7  Takahashi et al.
**Supplemental Data, FIGURE LEGENDS**

**Fig. S1** Molecular ion profiles of the permethylated derivatives of total desialylated PA-oligosaccharides from POT (A), POM (B), POA(hi) (C), and POA(lo) (D). The molecular compositions for the [M+H]$^+$ signals detected were listed in Table I.

**Fig. S2** Total ion chromatogram mass spectrometry (TIC) from GC-Mass spectrometry methylation analysis of the desialylated PA-oligosaccharides from POT (A), POM (B), POA(hi) (C), and POA(lo) (D). PMAA peaks were identified by retention time and EI-Mass spectrometry, as compared against authentic PMAA standards prepared from commercially available oligosaccharides.

**Fig. S3.** **HPLC profile on a DEAE column of pyridylamino N-glycans obtained from POT.** N-glycan fractions obtained by glycoamidase A digestion of POT were pyridylaminated and were first separated by on a DEAE column according to the sialic acid contents.

**Fig. S4.** **Two examples of HPLC profiles on amide-silica column of the separated N-glycans on the above ODS column.** Each of the separated N-glycans on the ODS column was applied individually to the third amide column. As examples, two main fractions N-IX and MS-V from ovotransferrin are illustrated.
Supplemental Data, Figure S1  Takahashi et al.
Supplemental Data, Figure S2  Takahashi et al.
N-Glycan structures from the major glycoproteins of pigeon egg white: Predominance of terminal Gal(\(\alpha\))1-4Gal
Noriko Takahashi, Kay-Hooi Khoo, Noriko Suzuki, James R. Johnson and Yuan C. Lee

*J. Biol. Chem.* published online April 2, 2001

Access the most updated version of this article at doi: 10.1074/jbc.M101380200

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