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Type and branched pattern of *N*-glycans and their structural effect on the chicken egg allergen ovotransferrin: a comparison with ovomucoid

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Abstract Ovotransferrin (OT), a multifunctional glycoprotein with defensive and protective activities, accounts for approximately 13 % of chicken egg white proteins and is known as a major egg-associated allergen along with ovomucoid (OM). In contrast to the well-characterized *N*-glycans of OM, the *N*-glycan structure of OT has not been reported. Here, using HPLC equipped with a fluorescence detector and mass spectrometric analysis in combination with exoglycosidase digestion, we investigated the *N*-glycan type and branched pattern of OT, and compared them with those of OM. The HPLC peak area was used to calculate the relative quantity (%) of each glycan. Seventeen *N*-glycans, including 11 glycans (1 core structure and 10 complex-type oligosaccharides), that commonly exist in ovotransferrin and ovomucoid were identified. Six characteristic glycans (2 truncated structures, 1 complex-type, and 3 hybrid-type oligosaccharides) in OT and eight characteristic glycans in OM were classified. OT contains the following branched complex-type structures: mono-(13.2 %), bi-(23.9 %), tri-(9.0 %), tetra-(2.7 %), and penta-(2.8 %) antennary oligosaccharides. However, OM contained mostly tri-(33.5 %) and penta-(31.2 %) antennary oligosaccharides. The *N*-glycan–containing bisecting *N*-acetylglucosamine comprised 43.4 % and 79.8 % of the total glycans in OT and OM, respectively. Moreover, using circular dichroism analysis, we observed that the secondary structure of the deglycosylated OT is quite different from that of the intact protein. To our knowledge, this is the first study to analyze *N*-glycans in OT in comparison with those of OM.

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Abbreviations

2-AB	2-aminobenzamide
ABS	α-sialidase from *Arthrobacter ureafaciens*
Ara	Arabinose
BTG	β-galactosidase from bovine testes
CD	Circular dichroism
Fuc	Fucose
Gal	Galactose
GalN	Galactosamine
GalNAc	*N*-acetylgalactosamine
Glc	Glucose
GlcN	Glucosamine
GlcNAc	*N*-acetylglucosamine
HPAEC	High-pH anion-exchange chromatography
Man	Mannose
MALDI-TOF/MS	Matrix-assisted laser desorption/ionization time-of-flight mass spectrometry
NeuAc	*N*-acetylneuraminic acid
NeuGc	*N*-glycolylneuraminic acid
NP-HPLC	Normal-phase high-performance liquid chromatography
OM	Ovomucoid
OT	Ovotransferrin
PAD	Pulsed amperometric detector
SDS-PAGE	Sodium dodecyl sulfate–polyacrylamide gel electrophoresis
SPH	Hexosaminidase from *Streptococcus pneumonia*
TFA	Trifluoroacetic acid
TFMS	Trifluoromethanesulfonic acid
Xyl	Xylose

Introduction

Ovotransferrin (OT) and ovomucoid (OM) are the main constituents of chicken egg white, accounting for the 13 % and 11 % of the total proteins, respectively [1]. These proteins are major allergens in egg white and commonly show allergenicity in patients with egg allergies. For example, Aabin *et al*. reported that 38 % and 32 % of patients with egg allergies are sensitive to OT and OM, respectively [2].

OT, a glycoprotein with a molecular weight of approximately 77.7 kDa, is composed of 686 amino acids, and contains an iron-binding region [3]. OT has 50 % homology with human transferrin and lactoferrin, but its isoelectric point and glycosylation pattern are quite different from those of human proteins [4, 5]. OT has been reported to have defensive and protective properties such as antimicrobial, antifungal, antiviral, immunomodulatory, anticancer, and antioxidative activities [3, 5]. Moreover, OM is a glycoprotein with a molecular weight of approximately 28 kDa having trypsin-inhibiting activity [6].

Most of protein allergens are glycosylated, with glycan probably contributing to their allergenicity, although its involvement in the allergenic response to these proteins has yet to be demonstrated definitively [7].

The glycosylation structure of OT is not well characterized, but it has been reported that OT has a single glycan chain composed of mannose (Man) and *N*-acetylglucosamine (GlcNAc) residues in its C-terminal domain [5]. In contrast to the paucity of knowledge regarding the structure of OT, glycan structure of OM has been determined in many studies. Yamashita *et al*. showed that OM mainly contains bi-, tri-, tetra-, and penta-antennary complex-type oligosaccharides harboring galactose (Gal) residues [8]. Sialylated oligosaccharides have also been found in OM [9].

Egg white proteins from other avian species also possess *N*-glycan. Duck OM contains mono-, bi-, tri-, and tetra-antennary complex-type oligosaccharides with Gal residues, but does not contain penta-antennary oligosaccharides [10]. Gull and pigeon OM contain not only bi-, tri-, tetra-, and penta-antennary complex-type oligosaccharides, but also the galabiose sequence (Galα1–4Gal) [11, 12]. The galabiose sequence is present in approximately two-thirds of Neoaves, but it is absent in Galloanserae such as chickens and ducks [13].

In this study, we analyzed the *N*-glycan structure of OT employing the high-pH anion-exchange chromatography (HPAEC), normal-phase high-performance liquid chromatography (NP-HPLC) of 2-aminobenzamide (2-AB) labeled glycans in combination with exoglycosidase digestion, and matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI-TOF MS). We found common and characteristic glycan structures between OT and OM, respectively. The relative quantities (%) of glycan types such as truncated, core, complex-, hybrid-, and high-mannose-types were calculated from the sum of the individual HPLC peak areas, and the five branched structures of mono-, bi-, tri-, tetra-, and penta-antennary complex-type oligosaccharides were classified. Finally, the comparison between intact and deglycosylated OT and OM was used to determine the structural effect of *N*-glycan on OT and OM.

Materials and methods

Sodium dodecyl sulfate-polyacrylamide gel electrophoresis

Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) was performed according to Laemmli [14] using 15 % acrylamide slab gel in the presence and absence of 2-mercaptoethanol. The gel was stained with Coomassie blue R-250. Molecular mass markers (6.5–200 kDa) were purchased from Bio-Rad (Hercules, CA, USA).

N-Glycan release by enzymatic deglycosylation

Enzymatic deglycosylation was performed according to Yagi *et al*. [15] with slight modifications. Briefly, the glycoproteins, OT and OM (Sigma, St. Louis, MO, USA) from chicken egg white were incubated in the presence of 40 μM trypsin (Sigma) and 40 μM chymotrypsin (Sigma) in 10 mM Tris–HCl buffer, pH 8.0, at 37 °C for 18 h. *N*-glycans were then released from glycopeptides after reaction with 0.1 mU glycoamidase A (Seikagaku Kogyo, Tokyo, Japan) in citrate-phosphate buffer, pH 5.0, at 37 °C for 18 h. To remove peptides and salts, a graphitized carbon cartridge (Alltech, Deerfield, IL, USA) was used according to Packer *et al*. [16]. The graphitized carbon cartridges were activated with 3 ml of 80 % acetonitrile containing 0.1 % trifluoroacetic acid (TFA) (Sigma), equilibrated and washed with 6 ml of 0.05 % TFA, and eluted with 2 ml of 25 % acetonitrile containing 0.05 % TFA. The released *N*-glycans were lyophilized and stored at −20 °C.

Monosaccharide composition analysis

N-glycans of OT and OM were incubated in 2 M TFA for 4 h at 100 °C for neutral and amino monosaccharides, and in 0.1 M HCl (Sigma) for 1 h at 80 °C for sialic acids. Monosaccharides were separated using a HPAEC system (Dionex, Sunnyvale, CA, USA) equipped with a CarboPac™ PA1 column (4× 250 mm) (Dionex) and an AminoTrap™ column (4×20 mm) (Dionex) working at an isocratic concentration of 20 mM NaOH (Fisher Scientific, Fair Lawn, NJ, USA) and at a flow rate of 0.5 ml/min at room temperature for 40 min for neutral and amino monosaccharides. Sialic acid analysis was carried out by a linear gradient of 70–300 mM sodium acetate in 100 mM NaOH for 10 min. Monosaccharides were detected by a pulsed amperometric detector (PAD). The concentration of

each monosaccharide for quantification was calculated from the calibration curve constructed using a standard mixture containing 2-fold dilutions from 1,600 pmol of each monosaccharide. Monosaccharides used in this study such as fucose (Fuc), Gal, glucose (Glc), Man, galactosamine (GalN), glucosamine (GlcN), xylose (Xyl), arabinose (Ara), *N*-acetylneuraminic acid (NeuAc), and *N*-glycolylneuraminic acid (NeuGc) were all purchased from Sigma.

2-AB labeling and solid-phase permethylation of *N*-glycans

The reducing ends of the released *N*-glycans were derivatized with 2-AB (Sigma) according to Bigge *et al*. [17] with slight modifications. Five mg of sodium cyanoborohydride (Sigma) and 5 mg of 2-AB were dissolved in dimethyl sulfoxide/acetic acid (7:3 v/v) solution. This solution was added to each lyophilized glycan sample, and the samples were incubated for 3 h at 65 °C. According to the procedure of Ruhaak *et al*. [18], 2-AB–labeled glycans were separated from excess fluorophore by microcrystalline cellulose (Sigma) column chromatography. The column was equilibrated and washed with 10 ml of n-butyl alcohol/acetic acid/ethanol (100:25:25, v/v/v) solution, and eluted with 4 ml of ethanol/75 mM ammonium bicarbonate (50:100, v/v). The glycans were lyophilized and stored at −20 °C. The released glycans for solid-phase permethylation was performed according to the procedure of Kang *et al*. [19]. Permethylated glycans were lyophilized and stored at −20 °C.

Exoglycosidase digestion of 2-AB–labeled glycans

2-AB–labeled glycans released from OT and OM were digested with exoglycosidases. All digestions were performed in 50 mM sodium phosphate buffer, pH 6.0, for 18 h at 37 °C. The exoglycosidases (ProZyme, Hayward, CA, USA) used were as follows: α-sialidase from *Arthrobacter ureafaciens* (ABS) 0.5 U/ml, β-galactosidase from bovine testes (BTG) 0.5 U/ml, and hexosaminidase from *Streptococcus pneumoniae* (SPH) 4 U/ml. To obtain exoglycosidase-treated glycans, a graphitized carbon cartridge (Alltech) was also used according to Packer *et al*. [16].

NP-HPLC analysis of 2-AB–labeled glycans

HPLC was performed using a Waters™ Alliance 2690 system equipped with a Waters™ 474 fluorescence detector (Waters, Milford, MA, USA). Purified 2-AB–labeled glycans were separated by NP-HPLC using a TSK-gel amide 80 column (4.6× 250 mm) (Tosoh, Tokyo, Japan) with slight modifications of the Guile *et al*. [20] method. Solvent A was 50 mM ammonium formate, pH 4.4, and solvent B was 100 % acetonitrile. Gradient conditions were as follows: time=0 min (t=0), 20 % solvent A (0.4 ml/min); t=132, 53 % solvent A (0.4 ml/min); t=135, 100 % solvent A (0.4 ml/min); t=137, 100 % solvent A (1 ml/min); t=142, 100 % solvent A (1 ml/min); t=145, 100 % solvent A (0.4 ml/min); t=145–160, 20 % solvent A (0.4 ml/min). Fluorescence was measured at 420 nm with excitation at 330 nm. The glucose unit (GU) values of the separated glycans were obtained from the retention time of the 2-AB–labeled glucose homopolymer ladder (Ludger, Oxfordshire, UK). Glycan structures were determined from GU values in comparison to the reference values in the "GlycoBase" (http://glycobase.nibrt.ie/glycobase/show_nibrt.action).

MALDI-TOF MS analysis of 2-AB–labeled or permethylated glycans

Dried 2-AB–labeled glycans were dissolved in water, and dried permethylated glycans were resuspended in ethanol/water (50:50, v/v) solution, respectively. A matrix solution was prepared as follows: acetonitrile/water (50:50, v/v) solution containing 2,5-dihydroxybenzoic acid (DHB) (Sigma) (10 mg/ml) for 2-AB–labeled glycans, and 1 mM sodium acetate containing DHB (10 mg/ml) for permethylated glycans. One microliter of sample solution and 1 μL of matrix solution were mixed and applied on the target spot of the analysis plate, and then dried in the vacuum desiccator. MALDI-TOF MS was carried out using the Ultraflex™ III system (Bruker Daltonik, Bremen, Germany) equipped with a Smartbeam laser (337 nm). Spectra were measured in the reflectron positive ionization mode.

Secondary structure determination of intact and deglycosylated forms of OT and OM using circular dichroism

Complete deglycosylation of OT and OM was performed using a chemical deglycosylation kit (Sigma) with trifluoromethanesulfonic acid (TFMS) hydrolysis according to the manufacturer's instructions. Deglycosylated OT and OM were dialyzed overnight against 10 mM phosphate-buffered saline (PBS) solution (pH 7.4). Far-UV circular dichroism (CD) spectra of intact and deglycosylated OT and OM were measured with a Chirascan plus CD spectrometer (Applied Photophysics, Leatherhead, UK). The samples were analyzed at 25 °C using a 0.02-cm quartz cuvette, and the corresponding spectra were recorded in the 195–260 nm wavelength region. Secondary structure components were estimated by *CDNN* CD spectra deconvolution software [21].

Results

SDS-PAGE and monosaccharide composition analysis

OT and OM were subjected to SDS-PAGE where they showed >98 % purity; therefore, no further purification process was

Table 1 Summary of monosaccharide composition analysis of *N*-glycans of OT and OM

Glycoprotein	Monosaccharide	Content (pmol)[a]	Relative amount (%)	Monosaccharide ratio[b]
OT				
	Gal	12.6±0.7	3.5	0.3±0.0
	Man	153.3±3.1	42.6	3.1±0.1
	GlcN	194.1±3.6	53.9	3.9±0.1
	NeuAc	0	0	0
OM				
	Gal	65.7±2.4	6.3	1.3±0.0
	Man	287.3±7.5	27.6	5.7±0.1
	GlcN	659.3±17.7	63.3	13.2±0.4
	NeuAc	28.7±0.5	2.8	0.6±0.0

The experiment was repeated three times, and data are expressed as mean ± standard deviation

[a] Content in 50 pmol of each glycoprotein

[b] Monosaccharide ratio is the number of moles of monosaccharide per mole of glycoprotein

carried out (data not shown). Monosaccharides were analyzed by the HPAEC-PAD method. Monosaccharide composition was identified by comparing the retention time of the monosaccharide peak in the standard. The monosaccharide ratio was calculated on the basis of the moles of monosaccharide per mole of glycoprotein. *N*-acetylgalactosamine (GalNAc) and GlcNAc were converted to GalN and GlcN, respectively, by de-*N*-acetylation during hydrolysis [22]. As indicated in Table 1, the monosaccharides of OT mainly comprised Man (42.6 %) and GlcN (53.9 %), with some Gal (3.5 %) but no NeuAc (0 %), while OM contained Gal (6.3 %), Man (27.6 %), GlcNAc (63.3 %), and NeuAc (2.8 %).

Structural analysis of *N*-glycan

N-glycans were released from OT and OM by glycoamidase A digestion. Each of the reducing ends of the released glycans was derivatized with 2-AB, and then the glycans were analyzed by NP-HPLC. In total, 17 and 19 glycans were detected in OT and OM, respectively (Fig. 1). The common structures (11) between OT and OM are presented as 1–11, and the characteristic structures of OT (6) and OM (8) are presented as T-a–f and M-a–h, respectively. The *N*-glycan structures were determined by their glucose unit (GU) values in comparison to a database of GU values of glycans analyzed by NP-HPLC. Additionally, their composition and structure were obtained from MALDI-TOF MS analysis. Their relative quantity (%) was obtained from the NP-HPLC area of each corresponding peak.

The 11 glycan structures that are common to OT and OM are listed in Table 2: 1 core structure (corresponding to peak 1) and 10 complex-type oligosaccharides (peaks 2–11). The six characteristic *N*-glycan structures of OT are listed in Table 3. OT specifically contains two truncated structures (peak T-a and T-b),

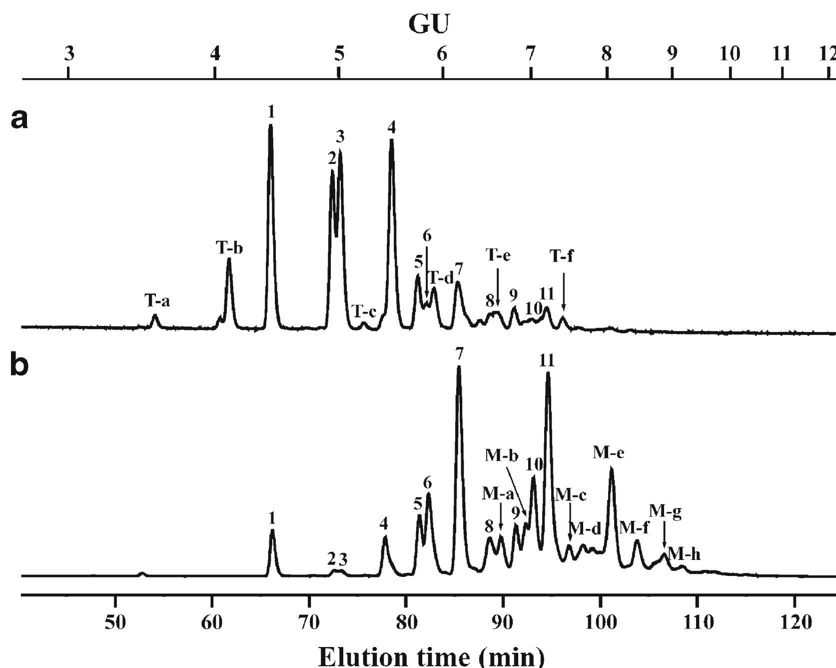

Fig. 1 HPLC chromatogram of *N*-glycans from (**a**) OT and (**b**) OM. The peaks showed the common structure of the two proteins (1–11). The characteristic structures of OT and OM are presented as T-a–f and M-a–h, respectively

Table 2 Summary of MS and HPLC data, and relative quantity (%) of the commonly found structures of 2-AB–labeled *N*-glycans from OT and OM

Peak	Proposed structure	MALDI-TOF MS m/z ([M + Na]$^+$)		GU			Relative quantity (%)	
		calculated	detected	reported	observed		OT	OM
					OT	OM		
1	Manα1-6 Manβ1-4GlcNAcβ1-4GlcNAc Manα1-3	1053.4	1053.4	4.40	4.42	4.44	17.3	3.3
2	GlcNAcβ1-2Manα1-6 Manβ1-4GlcNAcβ1-4GlcNAc Manα1-3	1256.5	1256.5	4.93	4.93	4.95	12.7	0.4
3	Manα1-6 GlcNAcβ1-4 – Manβ1-4GlcNAcβ1-4GlcNAc Manα1-3	1256.5	1256.5	4.97	5.00	5.01	16.5	0.4
4	GlcNAcβ1-2Manα1-6 Manβ1-4GlcNAcβ1-4GlcNAc GlcNAcβ1-2Manα1-3	1459.5	1459.6	5.45	5.48	5.42	19.2	3.5
5	GlcNAcβ1-2Manα1-6 GlcNAcβ1-4 – Manβ1-4GlcNAcβ1-4GlcNAc GlcNAcβ1-2Manα1-3	1662.6	1662.6	5.76	5.74	5.76	4.7	4.4
6	GlcNAcβ1-2Manα1-6 Manβ1-4GlcNAcβ1-4GlcNAc GlcNAcβ1-4 Manα1-3 GlcNAcβ1-2	1662.6	1662.6	5.90	5.83	5.85	1.8	7.5
7	GlcNAcβ1-2Manα1-6 GlcNAcβ1-4 – Manβ1-4GlcNAcβ1-4GlcNAc GlcNAcβ1-4 Manα1-3 GlcNAcβ1-2	1865.7	1865.7	6.14	6.16	6.17	5.6	17.4
8	GlcNAcβ1-6 Manα1-6 GlcNAcβ1-2 Manβ1-4GlcNAcβ1-4GlcNAc GlcNAcβ1-4 Manα1-3 GlcNAcβ1-2	1865.7	1865.7	6.53	6.51	6.51	0.8	3.5
9	GlcNAcβ1-6 Manα1-6 GlcNAcβ1-2 GlcNAcβ1-4 – Manβ1-4GlcNAcβ1-4GlcNAc GlcNAcβ1-4 Manα1-3 GlcNAcβ1-2	2068.8	2069.8	6.74	6.80	6.82	1.9	3.7
10	Galβ1-4 [GlcNAcβ1-2Manα1-6 GlcNAcβ1-4 – Manβ1-4GlcNAcβ1-4GlcNAc GlcNAcβ1-4 Manα1-3 GlcNAcβ1-2]	2027.8	2027.8	-[a]	7.01	7.03	1.6	8.6
11	GlcNAcβ1-6 GlcNAcβ1-4 – Manα1-6 GlcNAcβ1-2 GlcNAcβ1-4 – Manβ1-4GlcNAcβ1-4GlcNAc GlcNAcβ1-4 Manα1-3 GlcNAcβ1-2	2271.9	2272.9	-[a]	7.20	7.21	2.8	19.3

[a] The structure is not reported in Glycobase. Expected structures from MS data and exoglycosidase digestion are presented

Table 3 Summary of MS, HPLC data, and relative quantity (%) of the characteristic structures of 2-AB labeled *N*-glycans from OT

Peak	Proposed structure	MALDI-TOF MS m/z ([M + Na]$^{+}$)		GU		Relative quantity (%)
		calculated	detected	reported	observed	
T-a	Manα1-3or6Manβ1-4GlcNAcβ1-4GlcNAc	891.3	889.4	-[a]	3.57	1.0
T-b	GlcNAcβ1-2or4-[Manβ1-4GlcNAcβ1-4GlcNAc / Manα1-3or6]	1094.4	1094.5	-[a]	4.10	7.0
T-c	Manα1-6 \ GlcNAcβ1-4 – Manβ1-4GlcNAcβ1-4GlcNAc GlcNAcβ1-2Manα1-3 /	1459.5	1459.6	5.33	5.21	0.5
T-d	Manα1-3Manα1-6 \ Manβ1-4GlcNAcβ1-4GlcNAc GlcNAcβ1-2Manα1-3 /	1418.5	1418.6	6.00	5.90	3.8
T-e	Manα1-3Manα1-6 \ GlcNAcβ1-4 – Manβ1-4GlcNAcβ1-4GlcNAc GlcNAcβ1-4 \ Manα1-3 / GlcNAcβ1-2 /	1824.7	1824.7	6.62	6.59	1.8
T-f	Manα1-6 \ Manα1-6 \ Manα1-3 / GlcNAcβ1-4 – Manβ1-4GlcNAcβ1-4GlcNAc GlcNAcβ1-4 \ Manα1-3 / GlcNAcβ1-2 /	1986.7	1986.8	-[a]	7.41	1.0

[a] The structure is not reported in Glycobase. Expected structures from MS data and exoglycosidase digestion are presented

one complex-type (peak T-c) and three hybrid-type (peak T-d, T-e, and T-f) oligosaccharides that were absent in OM.

The eight characteristic *N*-glycan structures and their relative quantity of OM (M-a–h) are as follows: M-a, GlcNAcβ1-4(Galβ1-4GlcNAcβ1-2Manα1-3)(GlcNAcβ1-2Manα1-6)Manβ1-4GlcNAcβ1-4GlcNAc (3.0 %); M-b, GlcNAcβ1-4(GlcNAcβ1-2Manα1-3)(GlcNAcβ1-2(GlcNAcβ1-4)(GlcNAcβ1-6)Manα1-6)Manβ1-4GlcNAcβ1-4GlcNAc (3.2 %); M-c, oligosaccharide containing NeuAc (2.0 %, sialylated glycan whose structure was not determined); M-d, Galβ1-4|GlcNAcβ1-4(GlcNAcβ1-2(GlcNAcβ1-4)Manα1-3)(GlcNAcβ1-2(GlcNAcβ1-6)Manα1-6)Manβ1-4GlcNAcβ1-4GlcNAc (5.6 %); M-e, Galβ1-4|GlcNAcβ1-4[GlcNAcβ1-2(GlcNAcβ1-4)Manα1-3)(GlcNAcβ1-2(GlcNAcβ1-4)(GlcNAcβ1-6)Manα1-6]Manβ1-4GlcNAcβ1-4GlcNAc (10.0 %); M-f, NeuAcα2-3Galβ1-4|GlcNAcβ1-4[GlcNAcβ1-2(GlcNAcβ1-4)Manα1-3)(GlcNAcβ1-2(GlcNAcβ1-6)Manα1-6]Manβ1-4GlcNAcβ1-4GlcNAc (2.3 %); M-g, Galβ1-4[Galβ1-4)|GlcNAcβ1-4(GlcNAcβ1-2(GlcNAcβ1-4)Manα1-3)(GlcNAcβ1-2(GlcNAcβ1-4)(GlcNAcβ1-6)Manα1-6]Manβ1-4GlcNAcβ1-4GlcNAc (1.5 %), M-h, NeuAcα2-3Galβ1-4|GlcNAcβ1-4[GlcNAcβ1-2(GlcNAcβ1-4)Manα1-3)(GlcNAcβ1-2(GlcNAcβ1-4)(GlcNAcβ1-6)Manα1-6]Manβ1-4GlcNAcβ1-4GlcNAc (0.4 %) (data not shown). OM specifically contains complex-type oligosaccharides including three sialylated (peak M-c, M-f, and M-h) oligosaccharides that were confirmed by MALDI-TOF MS analysis of permethylated glycans (peaks M-f and M-h) (data not shown) and NP-HPLC analysis of ABS-treated glycans of OM (peaks M-c, M-f, and M-h) (data not shown) as well as by previous reports (peak M-h) [8, 9]. Previous reports on *N*-glycans of OM describe 11 major *N*-glycans [8, 9]. This study identified 8 new glycan structures (2, 3, 4, 6, 8 as shown in Table 2, and M-a, M-c, and M-f).

Exoglycosidase digestion of *N*-glycan

To confirm the glycan structure of OT, the 2-AB–labeled glycans were digested with various combinations of exoglycosidases, such as ABS, ABS+BTG, and ABS+BTG+SPH. Alteration of the HPLC chromatogram by treatment of OT with exoglycosidase is detailed in Fig. 2. Since it was previously reported that the removal of a β-linked Gal residue causes a shift of 0.8–0.9 GU, removal of a β-linked Man residue causes a shift of 0.7–0.9 GU, and removal of a β-linked GlcNAc

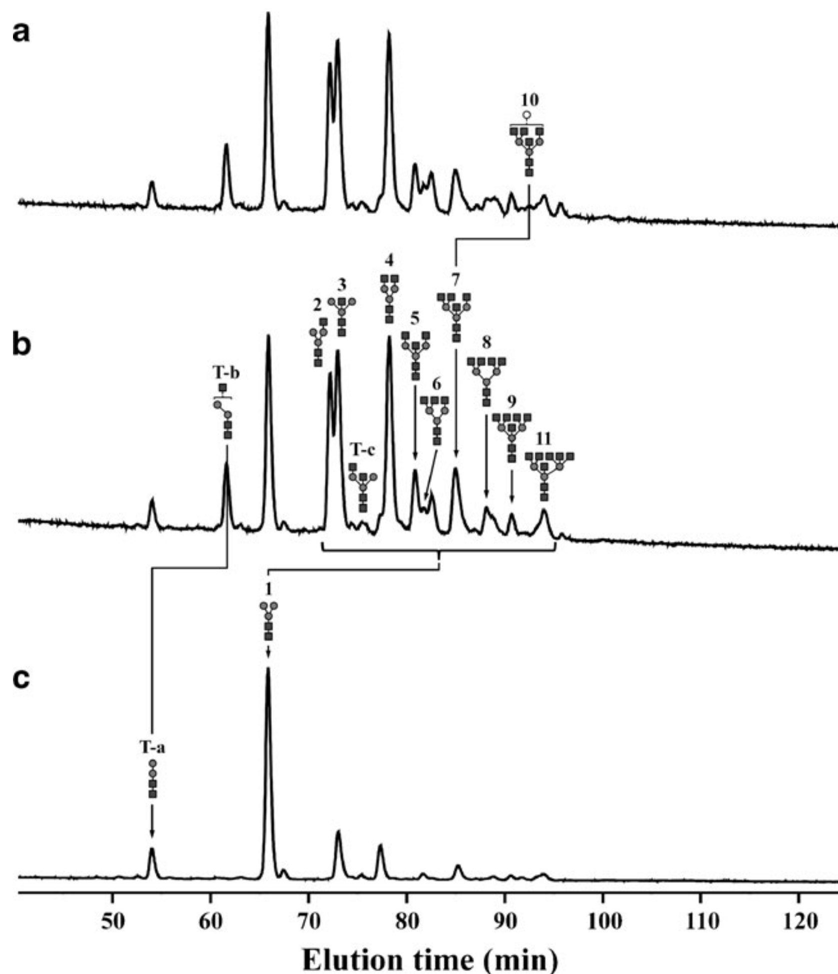

Fig. 2 HPLC chromatogram of exoglycosidase digestion of 2-AB–labeled *N*-glycans from OT digested with (**a**) ABS, (**b**) ABS+BTG, and (**c**) ABS+BTG+SPH. ABS removes α2-3,6,8-linked terminal NeuAc and NeuGc, BTG removes β1-3,4-linked terminal Gal, and SPH removes β1-2,3,4,6-linked terminal GlcNAc. The symbols for the glycan structures are as follows: ■ GlcNAc, ● Man, ○ Gal. The glycan structures were drawn using GlycoWorkbench [23]

residue causes a shift of 0.5 GU [23], this approach was applied to the sequential exoglycosidase digestion analysis. As shown in Fig. 2, ABS-treated OT resulted in no remarkable degradation (Compare Figs. 1a and 2a), indicating that OT does not contain NeuAc. Digestion with ABS and BTG resulted in Gal-attached glycans as evidenced by the shift of peak 10 to its galactosidase-treated glycan counterpart indicated by peak 7 (Fig. 2b). Treatment with ABS, BTG and SPH resulted in most peaks (2, 3, T-c, 4, 5, 6, 7, 8, 9, and 11) shifting to peak 1 [Manα1-3(Manα1-6)Manβ1-4GlcNAcβ1-4GlcNAc]. Peak T-b was shifted to peak T-a. The glycan structures in Fig. 2 were drawn using GlycoWorkbench [24].

Comparison of *N*-glycan type and branched pattern of OT and OM

The relative quantity (%) of the glycan types such as complex-, hybrid-, and high-mannose-types, was calculated from the sum of individual HPLC peaks, and classified into various branched patterns of mono-, bi-, tri-, tetra-, and penta-antennary complex-type oligosaccharides.

As shown in Fig. 3a, OT contains complex-type (68.1 %) and hybrid-type (6.6 %) oligosaccharides, but no high-mannose-type (0.0 %) oligosaccharides. OM consists almost entirely of complex-type (94.7 %) oligosaccharides, including three sialylated oligosaccharides. Core (17.3 %) and its truncated (8.0 %) structures of *N*-glycan remarkably existed in OT, whereas OM (3.3 % and 0.0 %, respectively) barely contained these structures. Various ratios of mono-, bi-, tri-, tetra-, and penta-antennary structures in complex-type oligosaccharides existed in each protein (Fig. 3b). OT contained five types of antennary complex-type structures as follows: mono-(13.2 %), bi-(23.9 %), tri-(9.0 %), tetra-(2.7 %), and penta-(2.8 %) antennary oligosaccharides. However, OM contained mostly tri-(33.5 %) and penta-(31.2 %) antennary, and very little mono-(0.4 %) antennary oligosaccharides. The relative quantity of *N*-glycan containing bisecting GlcNAc was 43.4 %, as calculated from the results of the ratio of the sum of ten peaks (3, 5, 7, 9, 10, 11, T-b, T-c, T-e, and T-f) area to the total peak area. However, 13 peaks (3, 5, 7, 9, 10, 11, M-a, M-b, M-d, M-e, M-f, M-g, and M-h) expressing bisecting GlcNAc existed in OM at a level of 79.8 % of the total (Tables 2 and 3).

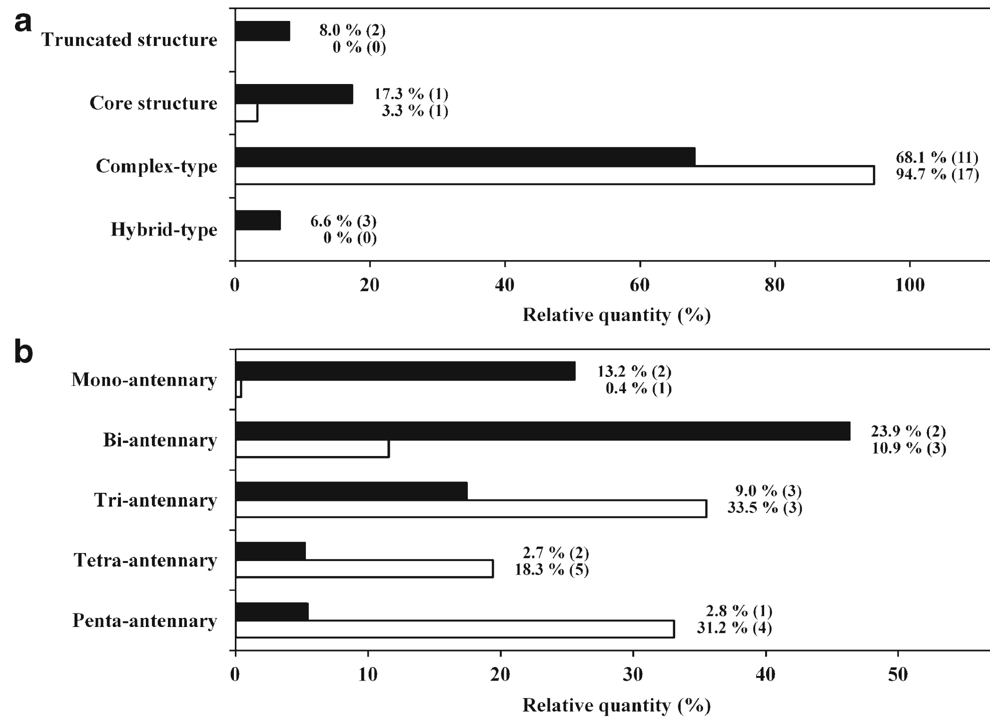

Fig. 3 Comparison of (**a**) glycan types from total oligosaccharides attached to OT (■) and OM (□) and (**b**) branched patterns of complex-type oligosaccharides from both common and specific oligosaccharides attached to OT (■) and OM (□), respectively. Relative quantities (%) are calculated from the sum of relative quantities of each glycan, and the number of glycans in each type is shown in parentheses

Secondary structure of intact and deglycosylated forms of OT and OM

The effects of *N*-glycans on the structures of OT and OM were investigated by measuring far-UV CD spectra of intact and deglycosylated OT and OM. Deglycosylated OT and OM were prepared using TFMS hydrolysis, and the absence of protein degradation as well as deglycosylation in each protein was confirmed by measuring the protein concentration, performing monosaccharide analysis and periodic acid-Schiff staining, and analyzing the SDS-PAGE gel (data not shown). It has previously been reported that TFMS has a minimal effect on the structural integrity of polypeptide chains in glycoproteins [25].

Far-UV CD spectra of intact and deglycosylated OT observed at 25 °C are shown in Fig. 4a. the contents of α-helix, β-sheets, β-turns, and random-coil structures in intact OT, estimated by *CDNN* CD spectra deconvolution software [21], were predicted at 23.5 %, 23.9 %, 17.2 %, and 35.4 %, respectively. In contrast, those in deglycosylated OT were estimated at 16.1 %, 33.1 %, 17.6 %, and 33.2 %, respectively. Intact OT clearly showed double minima at 208 nm and 222 nm, indicating the existence of α-helical structures [26], whereas α-helical structures decreased from 23.5 % to 16.1 % in deglycosylated OT (Fig. 4a). Far-UV CD spectra of intact and deglycosylated OM observed in the same experimental conditions were recorded, and the contents of α-helix, β-sheets, β-turns, and random-coil structures in intact OM (18.5 %, 30.1 %, 17.7 %, and 33.7 %, respectively) and deglycosylated OM (14.3 %, 34.7 %, 17.4 %, and 33.6 %, respectively) were estimated (Fig. 4b).

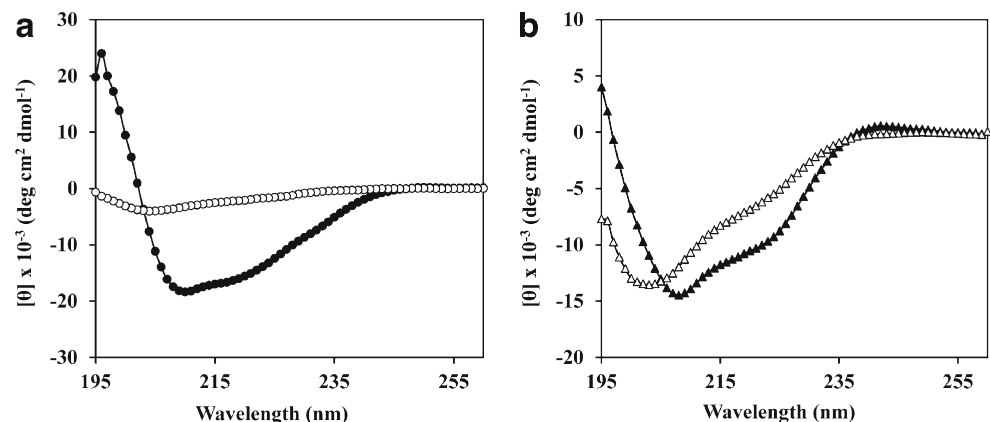

Fig. 4 Far-UV CD spectra of (**a**) intact OT (●) and deglycosylated OT (○) and (**b**) intact OM (▲) and deglycosylated OM (△) in 10 mM PBS (pH 7.4) at 25 °C in the 195–260 nm wavelength region

Discussion

In this study, we analyzed the *N*-glycan structures of OT through NP-HPLC and MALDI-TOF MS analysis, and compared them with those of commercially available OM from chicken egg white. All the analytical experiments were repeated three times, and exactly the same HPLC chromatogram and MS results were obtained. In previous studies, it was shown that sialic acid and Gal residues are absent in OT from chicken egg white [27]. However, in our investigation, one galactosylated glycan (peak 10) was found and confirmed by monosaccharide analysis of Gal (3.5 %) using HPAEC-PAD and exoglycosidase BTG digestion (Fig. 2a). As shown in Fig. 3 and Tables 2 and 3, chicken egg white proteins contain different types of glycans. OT contains five types of mono-, bi-, tri-, tetra-, and penta-antennary structures of complex-type oligosaccharides. In particular, the penta-antennary structure, which is seldom detected in human serum, is distinctively found in OT (2.8 %). These variously branched *N*-glycan structures result from mannosidase or *N*-acetylglucosaminyltransferases that exist in the oviduct [28]. It has been reported that *N*-glycans in chicken egg white proteins mainly consist of bisecting GlcNAc structures [29]; however, we quantitatively clearly confirmed that these bisecting structures accounted for the 43.4 % and 79.8 % of the total glycans in OT and OM, respectively. Moreover, our investigation confirmed that OT and OM do not contain the galabiose sequence.

Far-UV CD spectra were measured to estimate the secondary structures of intact and deglycosylated OT and OM. As shown in Fig. 4, OT showed double minima at 208 nm and 222 nm, indicating the existence of α-helical structures [30]. On the other hand, when carbohydrate moieties were removed by TFMS hydrolysis, the evidence of α-helical structures diminished. In the case of intact OM and deglycosylated OM, relatively little structural changes were observed. These results suggest that glycosylation of OT affects its secondary structures, particularly α-helical structures. It has been known that deglycosylation of glycoproteins such as OT decreases protein stability and denaturation enthalpy [31]; however, the secondary structural effect on OT has not been reported.

The possibility that protein glycosylation may contribute to allergenicity has been reported [7]. For example, investigations into the activities of IgG and IgE antibodies showed that the glycan moiety in the third domain of OM contributes to its allergenic properties [32, 33]. Moreover, recombinant glycoproteins with different glycosylations were less allergenic than the native ones [34]. In contrast, it is also known that the glycan of glycoproteins had no effect on their allergenicity [1], while non-glycosylated peptides derived from glycoproteins cause allergenicity [35]. Therefore, further research about the influence of glycosylation of glycoproteins such as OT on allergenicity is required.

Interestingly, the common glycan structures of OT and OM confirmed by the present study also exist in the glycans of ovalbumin, one of the major allergens in egg white as reported in earlier studies [36, 37], except for 2 glycans (6 and 8 in Table 2).

Unfortunately, the effects of glycan type and branched pattern on the function of OT demonstrated in this study are not well understood yet. However, many recent reports described glycans and allergenicity, such as the relation between the type and branching of *N*-glycan and their functions [38], classification of glycosylation pattern of common allergens [39], glycoside epitope in oligosaccharides of allergenic glycoproteins [40], or investigations on many protein allergens that are glycosylated describing how glycosylation may contribute to their allergenicity [7]. Our results provide useful information for elucidating the effect of *N*-glycan on the allergenicity of glycan-type and/or branched pattern in OT, and such research is currently underway in our lab. To our knowledge, this is the first report on the structural analysis of *N*-glycans of OT and its comparison with those of OM, along with the analysis of the relative quantity of *N*-glycosylated variants of OT and OM from chicken egg white.

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