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Structures of asparagine-linked oligosaccharides from hen egg-yolk antibody (IgY). Occurrence of unusual glucosylated oligo-mannose type oligosaccharides in a mature glycoprotein

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Asparagine-linked oligosaccharides present on hen egg-yolk immunoglobulin, termed IgY, were liberated from the protein by hydrazinolysis. After *N*-acetylation, the oligosaccharides were labelled with a UV-absorbing compound, *p*-aminobenzoic acid ethyl ester (ABEE). The ABEE-derivatized oligosaccharides were fractionated by anion exchange, normal phase and reversed phase HPLC, and their structures were determined by a combination of sugar composition analysis, methylation analysis, negative ion FAB-MS, 500 MHz ^{1}H-NMR and sequential exoglycosidase digestions. IgY contained monoglucosylated oligomannose type oligosaccharides with structures of Glcα1-3Man$_{7-9}$-GlcNAc-GlcNAc, oligomannose type oligosaccharides with the size range of Man$_{5-9}$GlcNAc-GlcNAc, and biantennary complex type oligosaccharides with core region structure of Manα1-6($\pm$GlcNAcβ1-4)(Manα1-3)Manβ1-4GlcNAcβ1-4($\pm$Fucα1-6)GlcNAc. The glucosylated oligosaccharides, Glc$_1$Man$_8$GlcNAc$_2$ and Glc$_1$Man$_7$GlcNAc$_2$, have not previously been reported in mature glycoproteins from any source.

Keywords: Asn-linked oligosaccharide, hen egg-yolk, IgY, immunoglobulin, *p*-aminobenzoic acid ethyl ester, HPLC, glucosylated oligosaccharide

Abbreviations: IgG, IgM, IgD, IgE, and IgA, immunoglobulin G, M, D, E, and A, respectively; IgY, egg-yolk antibody; ABEE, *p*-aminobenzoic acid ethyl ester; HPLC, high performance liquid chromatography; FAB-MS, fast atom bombardment mass spectrometry; Hex, hexose; HexNAc, *N*-acetylhexosamine; hCG, human chorionic gonadotropsin.

Hen serum has been known to contain three classes of immunoglobulins, namely: IgG, IgM and IgA. It has been shown that hen IgG differs from mammalian IgG in molecular size (larger), isoelectric point (more acidic), carbohydrate content (higher) and binding to mammalian complement and protein A [1–3]. Although the structures of oligosaccharide chains of human IgG have been extensively studied [4–6], there is limited information on this aspect in hen IgG. Comparison of the structures of oligosaccharides of hen IgG with those of human immunoglobulins is expected to contribute to an understanding of the evolution of the immune system. Although hen IgG is sometimes referred to as IgY [2], the nomenclature is not generally used. To distinguish serum antibody from that of egg yolk, we use the abbreviations IgY for egg-yolk antibody and IgG for serum in this paper.

The serum IgG has been shown to be preferentially transferred from the circulating blood of the hen to the egg-yolk as the means of maternal transfer of antibodies to the chick [7, 8]. Because this transfer is analogous to placental transfer of antibodies in mammals, it is of interest to compare the chemical and immunological properties of

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immunoglobulins from serum and egg-yolk in order to understand the maternal transfer of antibodies. It has been shown that egg-yolk immunoglobulin (IgY) is indistinguishable from serum IgG in antigen-binding capacity, dissociation rates of antibody–antigen complexes and molecular size [8]. However, there is no information on the carbohydrate moieties. Comparison of the oligosaccharide chains from egg-yolk IgY with those from serum IgG may provide knowledge for understanding the hen-to-chick antibody transfer. Recently, we reported a practical isolation procedure which provides highly pure IgY antibody in good yields [9].

As a step toward the elucidation of the structure and function of the oligosaccharide moieties of hen egg-yolk IgY and serum IgG, we have investigated the Asn-linked oligosaccharides derived from hen egg-yolk IgY by hydrazinolysis followed by conjugation with a UV-absorbing compound, *p*-aminobenzoic acid ethyl ester (ABEE). The present paper indicates that IgY contains unusual monoglucosylated oligomannose type oligosaccharide chains, which have not previously been found in any mature glycoprotein, in addition to oligomannose type and biantennary complex type oligosaccharides.

Materials and methods

Chemicals and enzymes

ABEE was purchased from Wako Pure Chemical Industries (Osaka, Japan). Sodium cyanoborohydride, α-methyl mannoside and ^2H_2O were purchased from Nacalai Tesque Inc. (Kyoto, Japan). Bovine pancreas ribonuclease B and ovalubumin were purchased from Sigma Chemical Co. (St. Louis, MO, USA), human chorionic gonadotropin (hCG) from Sankyo Zoki (Tokyo, Japan), and human IgG from ICN Immunobiologicals. Bio-Gel P-4 (200–400 mesh) was purchased from Bio-Rad (Richmond, CA, USA). *Aspergillus saitoi* α-mannosidase I was purified by the method of Amano and Kobata [10]. Jack bean meal β-galactosidase, β-*N*-acetylhexosaminidase and α-mannosidase, and bovine kidney α-L-fucosidase were purchased from Sigma. *Arthrobacter ureafaciens* neuraminidase was purchased from Nacalai Tesque Inc.

Standard oligosaccharides

Manβ1-4GlcNAcβ1-4GlcNAc-ABEE (M·GN·GN-ABEE) was prepared from goat kidney with β-mannosidosis [11]. Manα1-6(Manα1-3)Manβ1-4GlcNAcβ1-4GlcNAc-ABEE (M_3·GN·GN - ABEE),Manα1 - 6(Manα1 - 3)Manα1-6(Manα1 - 3)Manβ1 - 4GlcNAcβ1 - 4GlcNAc - ABEE(M_5·GN·GN - ABEE) and (Manα1 - 2)$_{1-4}$$Man_5$GlcNAcβ1-4GlcNAc - ABEE($M_{6-9}$·GN·GN - ABEE) were prepared from ribonuclease B and ovalbumin. Galβ1-4GlcNAcβ1-2Manα1 - 6(Galβ1 - 4GlcNAcβ1 - 2Manα1 - 3)Manβ1-4GlcNAcβ1-4GlcNAc - ABEE(G_2·GN_2·M_3·GN·GN-ABEE) and Galβ1-4GlcNAcβ1-2Manα1-6(Galβ1-4GlcNAcβ1 - 2Manα1 - 3)Manβ1 - 4GlcNAcβ1 - 4(Fucα1 - 6)GlcNAc-ABEE(G_2·GN_2·M_3·GN·F·GN-ABEE) were prepared from hCG. GlcNAcβ1 - 2Manα1 - 6(GlcNAcβ1 - 2Manα1 - 3)Manβ1-4GlcNAcβ1-4GlcNAc-ABEE(GN_2·M_3·GN·GN-ABEE) was prepared from G_2·GN_2·M_3·GN·GN-ABEE by jack bean β-galactosidase digestion. Galβ1-4GlcNAcβ1-2Manα1 - 6(GlcNAcβ1 - 2Manα1 - 3)Manβ1 - 4GlcNAcβ1 - 4(Fucα1 - 6)GlcNAc - ABEE(G′·GN_2·M_3·GN·F·GN-ABEE) and GlcNAcβ1 - 2Manα1 - 6(Galβ1 - 4GlcNAcβ1 - 2Manα1-3)Manβ1-4GlcNAcβ1-4(Fucα1-6)GlcNAc-ABEE (G·GN_2·M_3·GN·F·GN-ABEE) were prepared from human IgG. Oligosaccharides were liberated from glycoproteins and labelled with ABEE as described previously [12]. The ABEE-oligosaccharides were separated by Bio-Gel P-4 column chromatography and HPLC [12, 13], and their structures were confirmed by FAB-MS, exoglycosidase digestions and/or 500 MHz ^{1}H-NMR analyses.

Preparation of IgY

Immunoglobulin Y was purified to apparent homogeneity from hen egg-yolk by the procedure established previously [9].

Liberation of the Asn-linked oligosaccharides from IgY and preparation of ABEE derivatives

Oligosaccharides were liberated from IgY (100 mg) by hydrazinolysis/*N*-acetylation as described previously [12]. The crude reaction product thus obtained was derivatized with ABEE by reductive amination [12, 14] as follows. The oligosaccharide fraction was dissolved in 50 µl water. To this solution was added 200 µl of a reagent mixture freshly made by mixing 35 mg ABEE, 3.5 mg $NaBH_3CN$, 41 µl acetic acid and 350 µl methanol and heating the mixture at 80°C for 45 min. The reaction mixture was diluted with 1 ml water and extracted 5 times with 1 ml portions of diethyl ether. The ABEE-derivatized oligosaccharides recovered in the aqueous layer were separated from non-sugar materials by chromatography on a Pre-Sep C_{18} cartridge (Tessek, Mountain View, CA, USA) [12]. The ABEE-oligosaccharides were further purified on a Bio-Gel P-4 column (200–400 mesh, 1.0 cm × 45 cm) using 0.1 M pyridinium acetate buffer (pH 4.0) as eluent. All the fractions showing typical UV spectra for ABEE-oligosaccharide [12] were pooled and lyophilized.

High performance liquid chromatography

HPLC analyses were performed with a Shimadzu LC-6A liquid chromatograph. Anion exchange HPLC was performed on a TSKgel DEAE-5PW column (0.75 cm × 7.5 cm, Tosoh Co., Ltd.). The column was pre-equilibrated in 10 mM NaH_2PO_4 and the elution was performed isocratically for 10 min with 10 mM NaH_2PO_4 followed by a linear gradient to the final concentration of 170 mM NaH_2PO_4 over 40 min with a flow rate of 0.5 ml min^{-1} at ambient temperature. The ABEE-derivatized neutral oligosaccharides

were analysed either on a TSKgel Amide-80 column (0.46 cm × 25 cm, Tosoh) or a Wakosil 5C18-200 column (0.4 cm × 25 cm, Wako Pure Chemical Industries). A mixture of solvent A (acetonitrile:water, 9:1 by vol) and solvent B (acetonitrile:water, 1:9 by vol) was used for the elution of the Amide-80 column. The column was equilibrated with a mixture of solvents A:B, 80:20 and, after injection of the sample, elution was performed using a linear gradient to a ratio of solvents A:B, 50:50 over 60 min at a flow rate of 1.0 ml min^{-1} at 40°C. The ODS column was eluted with a mixture of solvent C (5% acetonitrile in 100 mM acetic acid) and solvent D (15% acetonitrile in 100 mM acetic acid). The column was equilibrated with a mixture of solvents C:D, 60:40. After injection of the sample, the elution was performed by a linear gradient to a mixture of solvents C:D, 40:60 over 60 min at a flow rate of 0.8 ml min^{-1} at 40°C. The elution was monitored at 304 nm [12].

Glycosidase digestion of oligosaccharides

ABEE-oligosaccharides (2–3 nmol) were digested in one of the following reaction mixtures at 37°C for 24 h under a toluene atmosphere. (1) 50 milliunits neuraminidase in 50 µl 0.1 M sodium acetate buffer, pH 4.5. (2) 100 milliunits β-galactosidase in 50 µl 0.05 M citrate-phosphate buffer, pH 3.5. (3) 0.5–2 units β-*N*-acetylhexosaminidase in 50 µl 0.1 M citrate-phosphate buffer, pH 5.0. (4) 1 unit α-mannosidase in 50 µl 0.1 M sodium acetate buffer, pH 4.5, containing 0.5 mM $ZnCl_2$. (5) 25 milliunits α-L-fucosidase in 0.05 M citrate-phosphate buffer, pH 5.5. (6) *A. saitoi* α-mannosidase I in 0.1 M sodium acetate buffer, pH 5.0. The reaction was terminated by heating at 100°C for 3 min. The reaction mixture was diluted with 1 ml water and the solution was applied to the Pre-Sep C_{18} cartridge. The cartridge was eluted stepwise with 10 ml each of water and water:acetonitrile (4:1). The product recovered in the latter fraction was analysed by HPLC.

Sugar composition analysis

Carbohydrate compositions were determined by gas chromatography of trimethylsilyl methyl glycosides obtained from ABEE-oligosaccharides by methanolysis in 1.5 M methanolic HCl (85°C, 16 h). Gas chromatography was performed on a CBP-1 fused silica capillary column (0.53 mm × 12 m, Shimadzu) using a Hewlett-Packard 5840A gas chromatograph.

Methylation analysis

ABEE-oligosaccharides were permethylated according to the method of Ciucanu and Kerek [15] and the methylated samples were hydrolysed, reduced and acetylated by the method of Liang *et al.* [16]. The partially methylated alditol acetates were analysed by gas chromatography/mass spectrometry on a Shimadzu model QP-1000 system. Separation was carried out on a CBP-1 fused silica capillary column (0.32 mm × 25 m, Shimadzu). Separation of 2,3,4,6-tetra-*O*-methylglucitol and 2,3,4,6-tetra-*O*-methylmannitol was performed on a Silar 10C WCOT fused silica capillary column (0.22 mm × 50 m, Chrompack Co.) with a column temperature of 180°C [17].

Fast atom bombardment mass spectrometry (FAB-MS)

Negative ion FAB-MS spectra were obtained with a JEOL JMS-HX100 mass spectrometer. An aqueous solution of an ABEE-oligosaccharide was mixed with glycerol on a stainless steel target, and the sample was bombarded by a xenon atom beam accelerated at a potential of 6 kV for analysis in the negative ion mode.

500 MHz ^{1}H-NMR spectrometry

ABEE-oligosaccharides were repeatedly exchanged in ^2H_2O (99.96% ^{2}H) with intermediate lyophilization. The 500 MHz ^{1}H-NMR spectra were obtained with a JEOL FX-500S FT NMR spectrometer operating in the Fourier-transform mode at a probe temperature of 25°C. Chemical shifts (δ, ppm) are given relative to sodium 4,4-dimethyl-4-silapentane 1-sulfonate, but were actually measured by reference to acetone in ^2H_2O (δ = 2.225 ppm). The signals were assigned by comparing the data of the spectra with those of standard ABEE-oligosaccharides, glucose-containing oligomannose type oligosaccharides [18–20], Asn-linked glycopeptides [21, 22], and 2-aminopyridine derivatives of oligosaccharides [6, 23, 24].

Results

Characterization of egg-yolk immunoglobulin

IgY was purified from egg-yolk as described previously [9]. The protein was dissociated into heavy and light chains with M_r 64 kDa and 28 kDa, respectively, on SDS PAGE [9]. The IgY preparation (1 mg) contained 11 nmol fucose, 110 nmol mannose, 35 nmol galactose, 15 nmol glucose, 45 nmol *N*-acetylglucosamine and 4 nmol sialic acid. This result suggests that IgY contained only Asn-linked oligosaccharides in which unique oligosaccharides containing glucose might be present.

Liberation and fractionation of oligosaccharides

Oligosaccharides were liberated from the IgY by hydrazinolysis/re-*N*-acetylation and then labelled with ABEE. The labelled oligosaccharides, purified by Pre-Sep C_{18} cartridge followed by gel filtration on Bio-Gel P-4, were separated into a neutral (N) and two acidic oligosaccharide fractions (SI and SII) by DEAE-5PW HPLC (Fig. 1). The molar ratios (%) of N, SI and SII were calculated as 60.2:32.3:7.5 on the basis of their UV-absorbance at 304 nm. The elution positions of SI and SII were identical with those of mono- and disialyl ABEE-oligosaccharides [13], respectively. Both acidic fractions were completely converted to neutral oligosaccharides by exhaustive sialidase digestion (data not shown), indicating that the acidic nature of these two

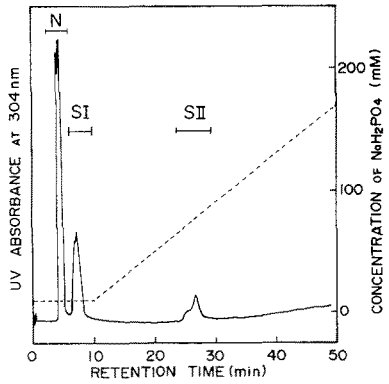


Figure 1. Ion-exchange HPLC of ABEE-oligosaccharides obtained from hen egg-yolk immunoglobulin (IgY). The ABEE derivatives of Asn-linked oligosaccharides derived from IgY were applied to a column of TSKgel DEAE-5PW (0.75 cm × 7 cm). The column was eluted isocratically with 10 mM NaH_2PO_4 for 10 min and then with a linear gradient from the same buffer composition to 170 mM NaH_2PO_4 over 40 min. Fractions indicated by bars were pooled. The elution positions of fractions N, SI and SII corresponded to those of the ABEE derivatives of neutral, monosialyl and disialyl oligosaccharides [13].

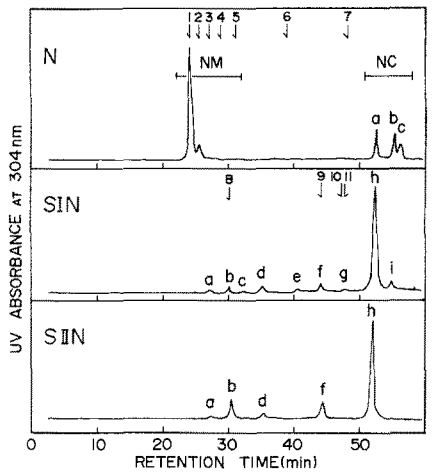


Figure 2. ODS/HPLC of neutral ABEE-oligosaccharides derived from hen egg-yolk immunoglobulin. ABEE-oligosaccharides in fraction N in Fig. 1 (N), sialidase-treated ABEE-oligosaccharides of fraction SI in Fig. 1 (SIN), and sialidase-treated ABEE-oligosaccharides of fraction SII in Fig. 1 (SIIN) were subjected to HPLC with a Wakosil 5C18-200 column (0.4 cm × 25 cm). The column was eluted with a mixture of acetonitrile and 100 mM acetic acid as described in the Materials and methods section. All peaks in group NM were pooled and subjected to fractionation by Amide-80 HPLC (see Fig. 3). Three peaks (NC—a–c) in group NC, 9 peaks of SIN, and 5 peaks of SIIN were collected and subjected to structural characterization. Arrows (↓) indicate the elution positions of standard ABEE-oligosaccharides: 1, $M_9 \cdot GN \cdot GN$-ABEE; 2, $M_8 \cdot GN \cdot GN$-ABEE; 3, $M_7 \cdot GN \cdot GN$-ABEE; 4, $M_6 \cdot GN \cdot GN$-ABEE; 5, $M_5 \cdot GN \cdot GN$-ABEE; 6, $M_3 \cdot GN \cdot GN$-ABEE; 7, $M \cdot GN \cdot GN$-ABEE; 8, $G_2 \cdot GN_2 \cdot M_3 \cdot GN \cdot GN$-ABEE; 9, $G_2 \cdot GN_2 \cdot M_3 \cdot GN \cdot F \cdot GN$-ABEE; 10, $G' \cdot GN_2 \cdot M_3 \cdot GN \cdot F \cdot GN$-ABEE; 11, $G \cdot GN_2 \cdot M_3 \cdot GN \cdot F \cdot GN$-ABEE.

fractions could be attributed to sialic acid residues. We refer to the desialylated ABEE-oligosaccharides from fractions SI and SII as SIN and SIIN, respectively.

The neutral ABEE-oligosaccharides (fraction N) obtained by DEAE-5PW HPLC were further fractionated by ODS HPLC. As shown in Fig. 2, the ABEE-oligosaccharides were separated into 2 groups, NM and NC. Elution positions of the peaks in group NM were identical to those of ABEE derivatives of oligomannose type oligosaccharides. In addition, all peaks in group NM were bound to a concanavalin A column and were eluted with 100 mM α-methyl mannoside (data not shown). Coupled with the methylation analysis (Table 1), these results indicate that the oligosaccharides in group NM were of the oligomannose type oligosaccharides. Because the separation of the peaks in the group NM on ODS/HPLC was incomplete, the material in all the peaks was pooled and fractionated by an Amide-80 HPLC, which can separate ABEE-oligosaccharides mainly based on their molecular sizes. As shown in Fig. 3, it was separated into 6 fractions (NM, a–f) with their elution positions corresponding to Hex_{5-10}GlcNAc-GlcNAc-ABEE. Based on their retention times, 3 ABEE-oligosaccharide peaks in group NC (NC, a–c) were assumed to be biantennary complex type ABEE-oligosaccharides. When the oligosaccharides in fractions SIN and SIIN were fractionated by ODS/HPLC, they were separated into 9 (SIN, a–i) and 5 (SNII, a, b, d, f and h) fractions, respectively (Fig. 2).

Structures of ABEE-oligosaccharides in group NM

The most predominant oligosaccharide (NM—f) contained glucose and mannose in a molar ratio of 1:8.5. Negative ion FAB-MS of this ABEE-oligosaccharide provided an $[M-H]^-$ ion at *m/z* 2192, corresponding to $Hex_{10}Hex\,NAc_2$-ABEE (Fig. 4). Methylation analysis (Table 1) of NM—f indicated 1 mol of non-reducing terminal glucose (2,3,4,6-tetra-*O*-methylglucitol), 2 mol of non-reducing terminal mannose (2,3,4,6-tetra-*O*-methylmannitol), 4 mol of C-2 substituted mannose, 1 mol of C-3 substituted mannose and 2 mol of C-3 and C-6 disubstituted mannose. By incubation with *A. saitoi* α-mannosidase I, which cleaves only Manα1-2Man linkages, the ABEE-oligosaccharide in NM—f showed a peak at the elution position of authentic $M_8 \cdot GN \cdot GN$-ABEE (loss of two mannose residues) on Amide-80 HPLC (Fig. 5). Together with the detection of a fragment ion which corresponds to $M-Hex_4$ in FAB-MS (Fig. 4), these results suggest a glucose was attached to the non-reducing end of the Manα1-2Manα1-2Manα1-3-branch of the typical $Man_9GlcNAc_2$-ABEE via an α(1-3)-linkage. The structure of the ABEE-oligosaccharide in NM—f was further confirmed by 500 MHz ^{1}H-NMR (Fig. 6, Table 2). The NMR spectrum of the compound is closely similar to that for $Glc_1Man_9GlcNAc_2$ [18–20], except for the chemical shift values of the H-1 and *N*-acetyl methyl protons of GlcNAc-2 and of H-1 and H-2 of Man-3, which showed

Table 1. Methylation analysis of ABEE-oligosaccharides obtained from IgY.

Methylated sugar	*Molar ratio*							
	NM[a]	SIN[b]	NM—d	NM—e	NM—f	NC—a	NC—b	NC—c
Fucitol								
2,3,4-Tri-*O*-methyl (1,5-di-*O*-acetyl)	–	0.74	–	–	–	0.90	0.87	1.22
Galactitol								
2,3,4,6-Tetra-*O*-methyl (1,5-di-*O*-acetyl)	–	2.00	–	–	–	2.10	1.26	–
Glucitol								
2,3,4,6-Tetra-*O*-methyl (1,5-di-*O*-acetyl)	2.80	–	0.67	0.75	1.09	–	–	–
Mannitol								
2,3,4,6-Tetra-*O*-methyl (1,5-di-*O*-acetyl)		–	2.33	2.25	1.91	–	–	–
3,4,6-Tri-*O*-methyl (1,2,5-tri-*O*-acetyl)	3.48	2.00	2.86	3.27	4.12	2.36	2.21	2.25
2,4,6-Tri-*O*-methyl (1,3,5-tri-*O*-acetyl)	0.92	–	0.64	0.73	0.97	–	–	–
2,4-Di-*O*-methyl (1,3,5,6-tetra-*O*-acetyl)	2.0	0.29	2.0	2.0	2.0	–	–	–
2-Mono-*O*-methyl (1,3,4,5,6-penta-*O*-acetyl)	–	0.71	–	–	–	1.0	1.0	1.0
2-*N*-Methylacetamido-2-deoxyglucitol[c]								
3,4,6-Tri-*O*-methyl (1,5-di-*O*-acetyl)	–	0.91	–	–	–	0.83	1.76	3.00
3,6-Di-*O*-methyl (1,4,5-tri-*O*-acetyl)	1.20	2.60	1.04	1.00	0.82	2.76	2.23	0.74

[a] The pooled sample of the group NM in Fig. 2.
[b] Desialylated sample of fraction SIN in Fig. 1.
[c] ABEE derivative of partially methylated 2-*N*-methylacetamido-2-deoxyglucitol could not be detected by the present method.

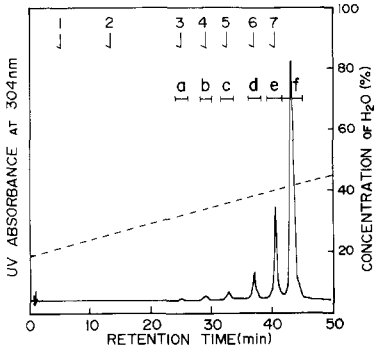


Figure 3. HPLC analysis of the pooled sample of ABEE-oligosaccharides in group NM. All peaks in group NM shown in Fig. 2 were pooled and subjected to HPLC analysis with a TSKgel Amide-80 (0.46 cm × 25 cm) column. The column was eluted with the mixture of acetonitrile and water described in the Materials and methods section. Six peaks indicated by bars were collected. Arrows (↓) indicate the elution positions of 1, M·GN·GN-ABEE; 2, M_3·GN·GN-ABEE; 3, M_5·GN·GN-ABEE; 4, M_6·GN·GN-ABEE; 5, M_7·GN·GN-ABEE; 6, M_8·GN·GN-ABEE and 7, M_9·GN·GN-ABEE.

an upfield shift of about 0.01 ppm by the introduction of ABEE at the reducing end. The signals at $\delta = 5.258$ ppm with coupling constant of 4.2 Hz, $\delta = 4.239$ ppm and $\delta = 5.039$ ppm coincided well with values for the H-1 of a Glcα1-3 residue, H-2 of Man-D_1 and H-1 of Man-D_1, respectively, in $Glc_1Man_9GlcNAc_2$-OH [18–20]. These results thus established the structure of the oligosaccharide in NM—f (Table 3).

Negative ion FAB-MS on NM—e provided an $[M-H]^-$ ion at *m/z* 2030, corresponding to $Hex_9HexNAc_2$-ABEE (Fig. 4). Composition analysis revealed this fraction to contain glucose and mannose in a molar ratio of 1:8.2. When the ABEE-oligosaccharide of peak NM—e was digested with *A. saitoi* α-mannosidase I, it yielded two peaks at the elution positions of authentic M_8·GN·GN-ABEE (loss of 1 mannose unit) and M_5·GN·GN-ABEE (loss of 4 mannose units) in the ratio of 43:57 on Amide-80 HPLC (Fig. 5). The latter compound was converted by jack bean α-mannosidase to an ABEE-oligosaccharide with the same mobility as authentic M·GN·GN-ABEE on Amide-80 and ODS HPLC (Fig. 5). In addition, methylation analysis indicated non-reducing terminal glucose and mannose, C-2 substituted mannose, C-3 substituted mannose and C-3 and C-6 disubstituted mannose as hexose derivatives (Table 1). These results suggest that fraction NM—e was a mixture of $Glc_1Man_8GlcNAcGlcNAc$-ABEE (43%) and $Man_9GlcNAcGlcNAc$-ABEE (57%). In negative ion FAB-MS of NM—e (Fig. 4), a fragment ion corresponding to $M-Hex_4$ was detected. This suggests that a glucose was attached to the non-reducing end of the Manα1-2Manα1-2Manα1-3 branch of the typical $Man_8GlcNAcGlcNAc$-ABEE. In addition to the signals found in the spectrum of oligosaccharide NM—f, the NMR spectrum of peak NM—e (Fig. 6 and Table 2) depicted 2 new anomeric proton signals corresponding to H-1 of Man-A without Man-D_2 (A′, $\delta = 5.083$ ppm) and of Man-B without Man-D_3 (B′, $\delta = 4.903$ ppm) [21–23]. From these results, the structures of the

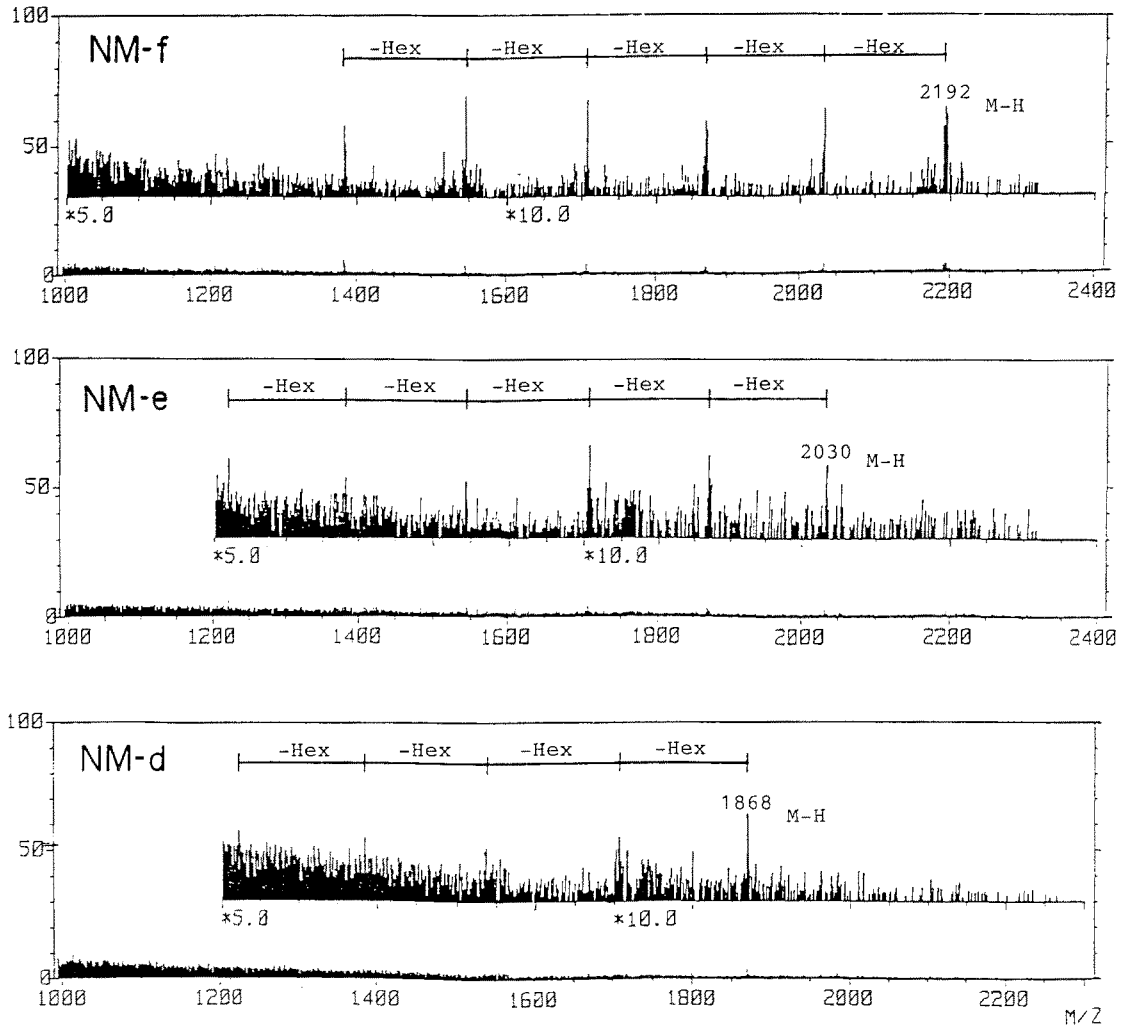


Figure 4. Negative ion FAB-MS of the ABEE-oligosaccharides in peak NM—d–f.

oligosaccharides present in fraction NM—e were established as shown in Table 3. Therefore, it was confirmed that hen egg-yolk immunoglobulin (IgY) contains unique glucosylated oligomannose type oligosaccharides of ±Manα1-2Manα1-6(∓Manα1-2Manα1-3)Manα1-6(Glcα1-3Manα1-2Manα1-2Manα1-3)Manβ1-4GlcNAcβ1-4GlcNAc which have not previously been reported as components of a mature glycoprotein.

The oligosaccharides in peak NM—d also contained a small amount of glucose. Negative ion FAB-MS provided an $[M-H]^-$ ion at *m/z* 1868, corresponding to $Hex_8HexNAc_2$-ABEE with the fragment ions corresponding to $M-Hex_{1\sim4}$. When oligosaccharides were digested with *A. saitoi* α-mannosidase I, about 60% of the oligosaccharides were hydrolysed to give a peak at the elution position of authentic $M_5 \cdot GN \cdot GN$-ABEE, whereas about 40% were resistant to the enzyme (Fig. 5). When the component eluting at the position of $M_5 \cdot GN \cdot GN$-ABEE was digested with jack bean α-mannosidase, a peak appeared at the position of authentic $M \cdot GN \cdot GN$-ABEE on Amide-80 and ODS HPLC (Fig. 5). These results together with methylation analysis data (Table 1), suggest that peak NM—d contained $Man_8GlcNAcGlcNAc$-ABEE (60%) and $Glc_1Man_7GlcNAcGlcNAc$-ABEE (40%). The NMR spectrum of the oligosaccharides provided the same anomeric proton signals as those found in the oligosaccharides NM—e (Fig. 6 and Table 2). From these results, the structures of oligosaccharides in NM—d were confirmed as shown in Table 3. The glucose-containing oligosaccharide, Manα1-6(Manα1-3)Manα1-6(Glcα1-3Manα1-2Manα1-2Manα1-3)Manβ1-4GlcNAcβ1-4GlcNAc, has not previously been found in any mature glycoprotein, nor have the similar oligosaccharides in NM—e.

Peak NM—c was eluted at the position of authentic $M_7 \cdot GN \cdot GN$-ABEE on the Amide-80 HPLC (Fig. 3), and the negative ion FAB-MS provided an $[M-H]^-$ ion at *m/z* 1706 corresponding to $Hex_7HexNAc_2$-ABEE (data not shown). When the oligosaccharides in this fraction were digested with *A. saitoi* α-mannosidase I, 2 mannose residues were removed from about 98% of the oligosaccharides,

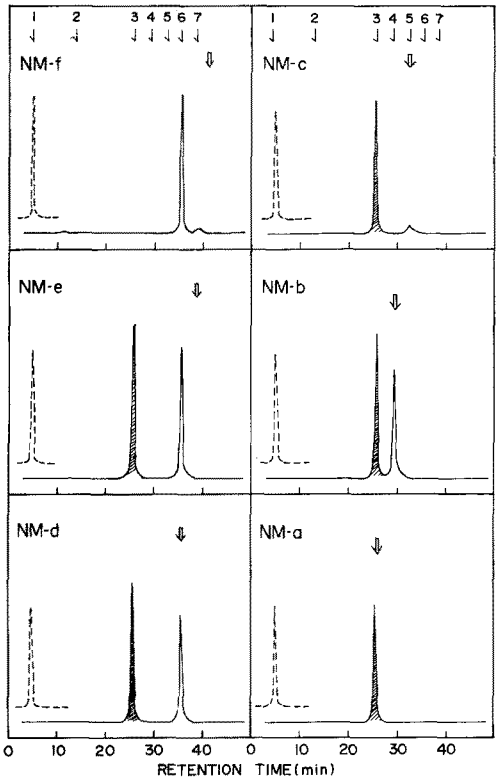


Figure 5. HPLC analysis of the digestion products of ABEE-oligosaccharides in peaks NM—a–f by *A. saitoi* α-mannosidase I. The ABEE-oligosaccharides in peaks NM—a–f (see Fig. 3) were digested with *A. saitoi* α-mannosidase I and the products were subjected to HPLC analysis on a TSKgel Amide-80 column under the conditions described in Fig. 3. The arrows (↓) indicate elution positions of standard compounds described in Fig. 3. Open arrows (⇓) indicate the elution positions of intact ABEE-oligosaccharides. The peak fractions eluted at the position of $M_5 \cdot GN \cdot GN$-ABEE (shaded area) were pooled. When this was subjected to jack bean α-mannosidase digestion, a peak corresponding to $M \cdot GN \cdot GN$-ABEE on Amide-80 and ODS HPLC (peak shown by dotted line) was observed.

yielding a peak corresponding to authentic $M_5 \cdot GN \cdot GN$-ABEE (Fig. 5). This product was converted to a component with the same mobility as authentic $M \cdot GN \cdot GN$-ABEE by jack bean α-mannosidase digestion (Fig. 5). These results indicated that NM—c contained no glucosylated oligosaccharide but only oligomannose type oligosaccharide with 6 α-mannosyl residues (Table 3).

Peak NM—b was eluted at the position of authentic $M_6 \cdot GN \cdot GN$-ABEE on the Amide-80 HPLC (Fig. 3) and FAB-MS provided an $[M-H]^-$ ion at m/z 1544, corresponding to $Hex_6HexNAc_2$-ABEE (data not shown). When the oligosaccharides were digested with *A. saitoi* α-mannosidase I, 1 mannose residue was removed from about 60% of the oligosaccharides and a peak appeared at the elution position of authentic $M_5 \cdot GN \cdot GN$-ABEE (Fig. 5). The product was converted to a component with the same mobility as authentic $M \cdot GN \cdot GN$-ABEE by jack bean

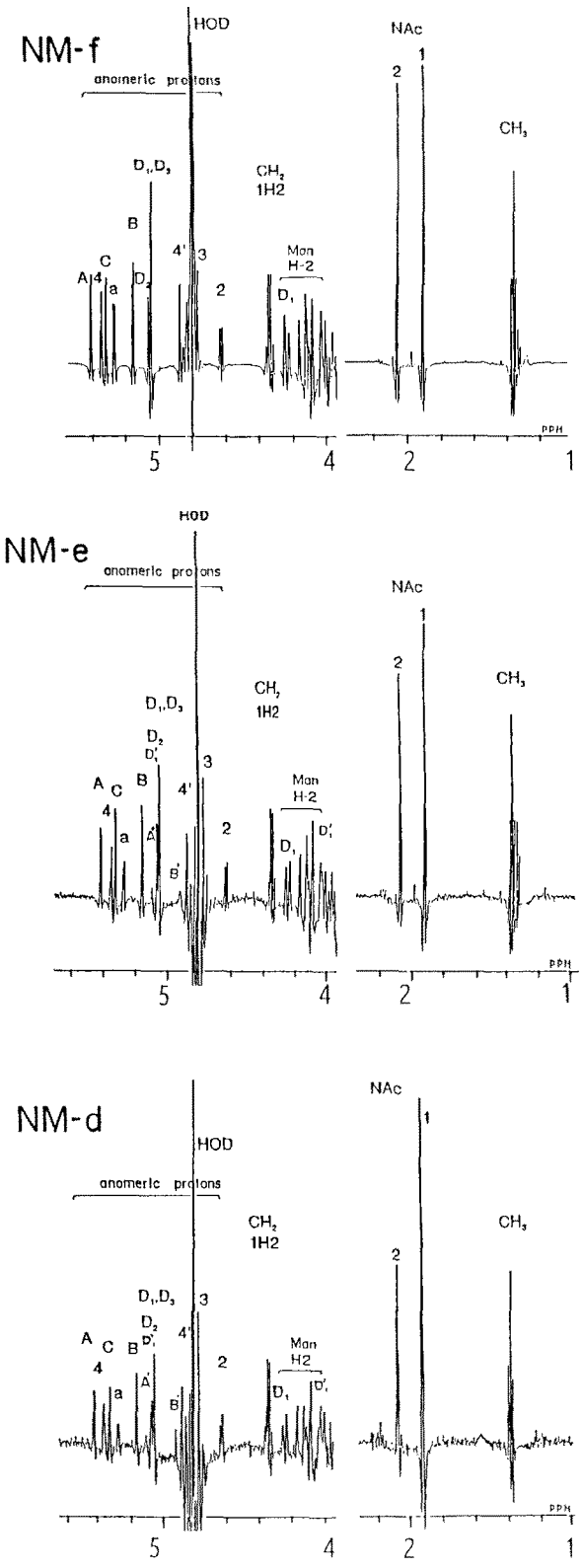
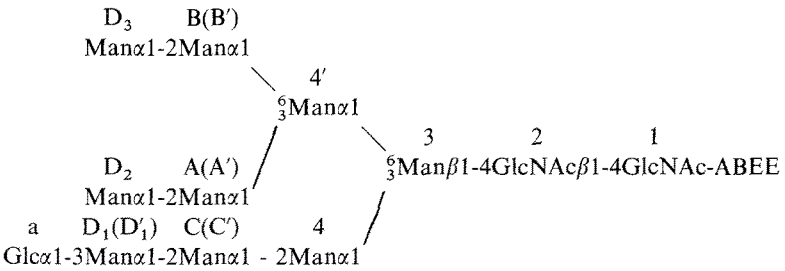


Figure 6. 500 MHz ^{1}H-NMR spectra of anomeric and methyl protons of ABEE-oligosaccharides in peaks NM—d–f from IgY. Monosaccharide residues are numbered similarly to those shown in Table 2. Spectra were recorded in ^2H_2O solution at 25°C. The signal at $\delta = 1.356$ ppm is due to the methyl protons of ABEE.

Table 2. ^1H chemical shifts of structural-reporter groups for the ABEE derivatives of oligosaccharides.



Reporter group	*Residue*	*Chemical shifts*[a] (ppm) *in*		
		NM—f $GlcMan_9GN_2$-ABEE	NM—e $GlcMan_8GN_2$-ABEE Man_9GN_2-ABEE	NM—d $GlcMan_7GN_2$-ABEE Man_8GN_2-ABEE
H-1	GlcNAc-2	4.619	4.617	4.617
	Man-3	4.762	4.763	ND[b]
	Man-4	5.337	5.336	5.338
	Man-4'	4.867	4.865	4.867
	Man-A, (-A')[c]	5.403	5.402, (5.083)	5.403, (5.082)
	Man-B, (-B')[c]	5.144	5.143, (4.903)	5.144, (4.904)
	Man-C	5.309	5.308	5.307
	Man-D_1, (-D'_1)[c]	5.039	5.037, (5.052)	5.037, (5.056)
	Man-D_2	5.054	5.052	5.056
	Man-D_3	5.039	5.037	5.037
	Glc-a	5.258	5.256	5.256
H-2	GlcNAc-1	4.342	4.336	4.338
	Man-3	4.216	4.215	4.215
	Man-4	4.091	4.090	4.090
	Man-4	4.152	4.151	4.151
	Man-A, (-A')[c]	4.112	4.108, (4.068)	4.109, (4.069)
	Man-B, (-B')[c]	4.016	4.018, (3.990)	4.017, (3.984)
	Man-C	4.112	4.108	4.109
	Man-D_1, (-D'_1)[c]	4.239	4.238, (4.068)	4.238, (4.069)
	Man-D_2	4.070	4.068	4.069
	Man-D_3	4.070	4.068	4.069
NAc	GlcNAc-1	1.906	1.907	1.909
	GlcNAc-2	2.057	2.055	2.057

[a] Data were obtained at 500 MHz in ^2H_2O solution at 25°C.
[b] Value could not be determined merely by inspection of the spectrum.
[c] A', B' and D'_1 mean the mannose and glucose residue without Man-D_2, Man-D_3 and Glc-a, respectively.

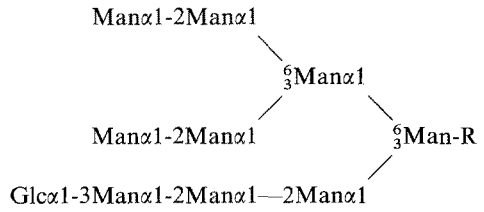
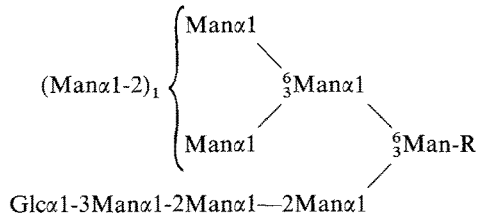
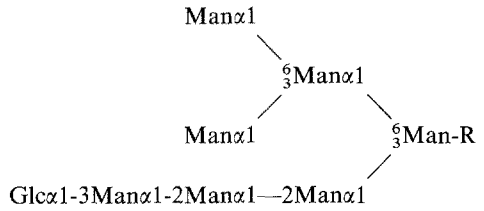
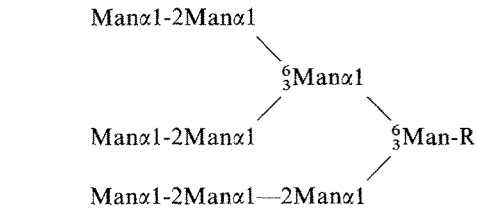
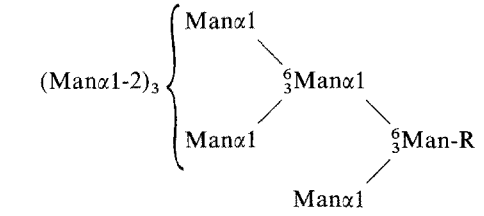
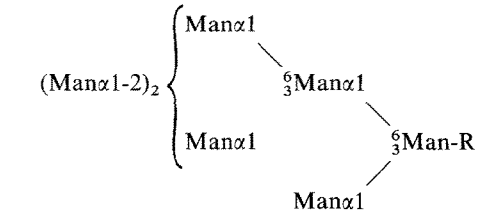
α-mannosidase digestion (Fig. 5). These results suggest that the component susceptible to *A. saitoi* α-mannosidase I was an oligomannose type oligosaccharide with 5 α-mannosyl residues (Table 3). Structural characterization of the component resistant to *A. saitoi* α-mannosidase I could not be performed due to the limited amount of sample.

Peak NM—a, eluted at the position of authentic M_5·GN·GN-ABEE, was resistant to *A. saitoi* α-mannosidase I (Fig. 5). When oligosaccharides in this fraction were digested with jack bean α-mannosidase, the oligosaccharides were deprived of 4 mannose residues and a peak appeared at the elution position of authentic M·GN·GN-ABEE (Fig. 5). These results indicate that peak NM—a contained only an oligomannose type oligosaccharide containing 4 α-mannosyl residues (Table 3).

Structure of the oligosaccharides in group NC

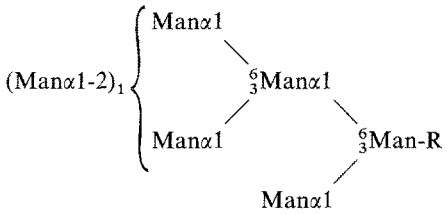
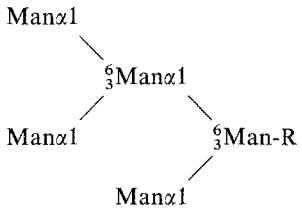
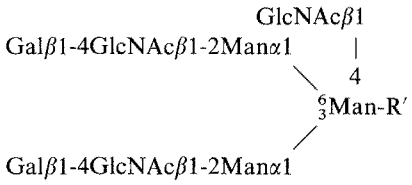
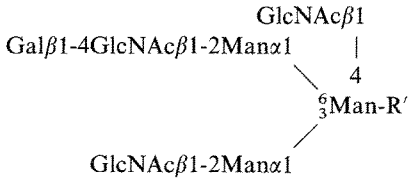
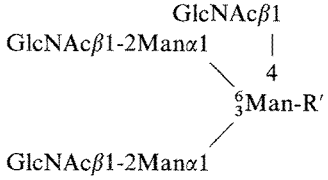
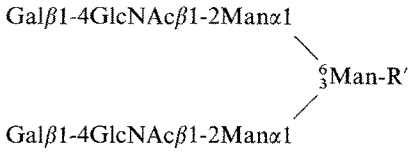
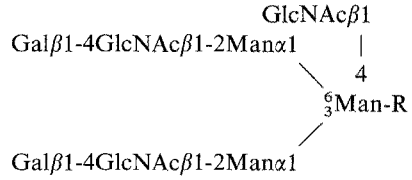
Negative ion FAB-MS of NC—a–c gave $[M-H]^-$ ions at *m/z* 2137, corresponding to $Hex_5FucHexNAc_5$-ABEE, 1975 for $Hex_4FucHexNAc_5$-ABEE, and 1813 for $Hex_3FucHexNAc_5$-ABEE, respectively. Together with sugar composition analysis (data not shown), methylation analysis (Table 1) and HPLC characteristics (Fig. 2) of the oligosaccharides, these results suggest that fractions NC—a–c were biantennary complex type oligosaccharides with a bisecting *N*-acetylglucosamine unit and a fucose residue, and contain

Table 3. Proposed structures of Asn-linked oligosaccharides obtained from hen egg-yolk immunoglobulin (IgY).

Structures[a]	*Fraction No.*[b]	*Relative amount*[c]		
		N	SIN	SIIN
	NM—f	21.4	–	–
	NM—e	4.4	–	–
	NM—d	1.3	–	–
	NM—e	5.7	–	–
	NM—d	1.9	–	–
	NM—c	0.7	–	–

(*table continues on next page*)

Table 3 (*continued*)

Structures[a]	*Fraction No.*[b]	*Relative amount*[c]		
		N	SIN	SIIN
	NM—b	0.2	–	–
	NM—a	0.1	–	–
	NC—a SIN—h SIIN—h	9.2	25.7	5.8
	NC—b SIN—i	8.5	1.8	–
	NC—c	6.8	–	–
	SIN—f SIIN—f	–	1.6	0.8
	SIN—d SIIN—d	–	1.4	0.2

(*table continues on next page*)

Table 3 (*continued*)

Structures[a]	*Fraction No.*[b]	*Relative amount*[c]		
		N	SIN	SIIN
Galβ1-4GlcNAcβ1-2Manα1 \ $^{6}_{3}$Man-R / Galβ1-4GlcNAcβ1-2Manα1	SIN—b	–	0.8	
	SIIN—b			0.7
Uncharacterized oligosaccharides[d]	NM—b	<0.1		
	SIN—a, c, e, g		1.0	
	SIIN—a			<0.1

[a] R = -GlcNAcβ1-4GlcNAc, R′ = -GlcNAcβ1-4(Fucα1-6)GlcNAc.
[b] See Figs 2 and 3.
[c] Relative amounts are expressed as percentage of the total oligosaccharides.
[d] Structures could not be determined due to the limited amount available.

2, 1 and no galactose residues, respectively, at their non-reducing ends.

In order to confirm these structures, samples were subjected to analysis by ^{1}H-NMR. Chemical shifts of the structural reporter-group protons of samples and reference compounds are summarized in Table 4. The presence of *N*-acetyl methyl proton signals for GlcNAc-1 (δ = 1.931–8 ppm) and GlcNAc-2 (δ = 2.069–2.074 ppm) and H-1 signals for GlcNAc-2 (δ = 4.680–2 ppm), Man-3 (δ = 4.690–1 ppm), Man-4 (δ = 5.052–5 ppm) and Man-4′ (δ = 4.989–4.999 ppm) indicate that all saccharides in the group NC contained the core Manα1-6(Manα1-3)Manβ1-4GlcNAc β1-4GlcNAc-ABEE structure. As well as these signals, all the fractions showed signals at δ = 2.065–6 and δ = 4.456–63 ppm due to the *N*-acetyl methyl protons and H-1 (β-anomer) of the bisecting *N*-acetylglucosamine. Chemical shifts of the methyl proton at δ = 1.120–2 ppm and H-1 at δ = 4.849–4.854 ppm revealed that a fucose residue was attached to the reducing end *N*-acetylglucosamine via an α(1-6)-linkage [6, 24]. In the spectrum of NC—a, signals corresponding to Gal-6, Gal-6′, GlcNAc-5, and GlcNAc-5′ were observed, indicating that the oligosaccharide NC—a possessed the structure shown in Table 3. In the spectrum of NC—b, the signal attributed to 1 galactose residue was observed at δ = 4.466 ppm. In addition, the H-1 signal of Man-4′ was observed slightly downfield (0.01 ppm) compared with that in NC—c. These results indicated that the oligosaccharide in peak NC—b contained 1 galactose residue, which was linked to the Manα1-6Man branch [6, 24] as shown in Table 3.

In the spectrum of NC—c, no anomeric proton signals due to galactose were detected and signals due to GlcNAc-5 and -5′ were significantly shifted upfield. By comparing these results with those of 2-aminopyridine derivatives of oligosaccharides [6, 24], the structure of NC—c in Table 3 was confirmed.

Structures of oligosaccharides in fractions SIN and SIIN

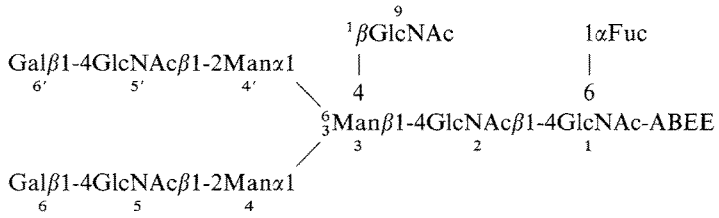
HPLC of the fractions SIN and SIIN on a Wakosil 5C18 column is shown in Fig. 2. Since the structures of the oligosaccharides in peaks SIIN—b, d, f, and h were the same as those of SIN—b, d, f, and h, respectively, data obtained from the analysis of the SIN fraction will be given below.

Peak SIN—h, the most predominant component derived from the acidic fraction, eluted at the same position as NC—a (Fig. 2). In addition, the NMR spectrum of the oligosaccharide in peak SIN—h was identical with that in NC—a (data not shown). The oligosaccharide structure was further confirmed by sequential exoglycosidase digestion (Fig. 7).

Oligosaccharide structures of SIN—b, d and f in Table 3 were proposed on the following basis; linkages indicated were obtained from methylation analysis data of the total oligosaccharide mixture SIN (Table 1) because they were the most probable even though the amount available was limited. Peak SIN—b eluted at the position of authentic $G_2 \cdot GN_2 \cdot M_3 \cdot GN \cdot GN$-ABEE. When the oligosaccharide in peak SIN—b was digested with β-galactosidase and analysed by ODS/HPLC, a peak appeared with the same mobility as authentic $GN_2 \cdot M_3 \cdot GN \cdot GN$-ABEE (Fig. 7). The digest was sequentially digested with β-*N*-acetylhexosaminidase (0.5 units) followed by jack bean α-mannosidase prior to subjecting the digests to HPLC analysis. A peak with the same mobility as that of authentic $M \cdot GN \cdot GN$-ABEE was obtained (Fig. 7). Based on these results and methylation analysis (Table 1), the structure of the oligosaccharide in peak SIN—b in Table 3 was proposed.

Peak SIN-d eluted at the position of a reference biantennary oligosaccharide with bisecting *N*-acetylglucosamine, prepared by α-L-fucosidase digestion of oligosaccharide NC—a (Fig. 7). When the oligosaccharide in peak SIN—d was sequentially digested with β-galactosidase,

Table 4. ^1H chemical shifts of anomeric protons and methyl protons for the ABEE derivatives oligosaccharides.



Reporter group	*Residue*	*Chemical shifts*[a] (ppm) *in*				
		NC—a $G_2 \cdot GN_3 \cdot M_3 \cdot GN \cdot F \cdot GN$-ABEE	NC—b $G \cdot GN_3 \cdot M_3 \cdot GN \cdot F \cdot GN$-ABEE	NC—c $GN_3 \cdot M_3 \cdot GN \cdot F \cdot GN$-ABEE	$G_2 \cdot GN_2 \cdot M_3 \cdot GN \cdot GN$-ABEE	$G_2 \cdot GN_2 \cdot M_3 \cdot GN \cdot F \cdot GN$-ABEE
H-1	GlcNAc-2	4.680	4.682	4.682	4.625	4.700
	Man-3	4.690	4.691	4.691	4.762	4.745
	Man-4	5.052	5.055	5.053	5.117	5.118
	Man-4′	4.998	4.999	4.989	4.925	4.924
	GlcNAc-5	4.565	4.551	4.545	4.576	4.577
	GlcNAc-5′	4.565	4.567	4.534	4.576	4.577
	Gal-6	4.463	–	–	4.467	4.464
	Gal-6′	4.463	4.466	–	4.467	4.464
	GlcNAc-9	4.463	4.457	4.456	–	–
	Fuc	4.849	4.854	4.853	–	4.850
NAc	GlcNAc-1	1.938	1.935	1.931	1.907	1.944
	GlcNAc-2	2.074	2.071	2.069	2.068	2.074
	GlcNAc-5	2.056	2.055	2.055	2.052	2.054
	GlcNAc-5′	2.033	2.030	2.038	2.043	2.044
	GlcNAc-9	2.066	2.065	2.065	–	–
Me	Fuc	1.120	1.121	1.122	–	1.129

[a] Data were obtained at 500 MHz in ^2H_2O solution at 25°C.

β-*N*-acetylhexosaminidase (2 units) and jack bean α-mannosidase, an ABEE derivative with the same retention time as authentic M·GN·GN-ABEE, uses obtained (Fig. 7). Thus, the oligosaccharide in peak SIN—d was proposed as a biantennary complex type oligosaccharide with bisecting GlcNAc (Table 3).

Peak SIN—f eluted at the position of authentic $G_2 \cdot GN_2 \cdot M_3 \cdot GN \cdot F \cdot GN$-ABEE, and an ABEE derivative appeared with the same retention time as $G_2 \cdot GN_2 \cdot M_3 \cdot GN \cdot GN$-ABEE, by α-fucosidase digestion (Fig. 7). Further sequential exoglycosidase degradation of the digest produced the same results as those described for oligosaccharide SIN—b (Fig. 7).

Peaks SIN—c, g and i are minor components in IgY: they eluted at the same positions as authentic $G \cdot GN_2 \cdot M_3 \cdot GN \cdot GN$-ABEE, $G \cdot GN_2 \cdot M_3 \cdot GN \cdot F \cdot GN$-ABEE and oligosaccharide NC—b, respectively (Fig. 2). Further analysis could not be performed on these fractions because of the lack of an adequate amount of sample. For the same reason, oligosaccharide structures in SNI—a and e were also not determined.

In summary, oligosaccharide structures in SIN and SIIN, derived, respectively, from mono- and disialyl oligosaccharide fractions, were proposed as shown in Table 3.

Discussion

We have studied the structures of the Asn-linked oligosaccharides derived from hen egg-yolk immunoglobulin (IgY) by hydrazinolysis followed by conjugation with ABEE. Among the total ABEE-derivatized oligosaccharides from IgY, approximately 60.2% were neutral, 32.3% monosialo, and 7.5% disialo oligosaccharides. Structures of oligosaccharides were determined after sialidase treatment as shown in Table 3. Our results showed that IgY contained three types of oligosaccharides: monoglucosylated oligomannose type oligosaccharides, $Glc_1Man_{7-9}GlcNAc_2$ (about 27.1% of total IgY oligosaccharides) with structures of ±Manα1 - 2Manα1 - 6(±Manα1 - 2Manα1 - 3)Manα1 - 6(Glcα1-3Manα1-2Manα1-2Manα1-3)Manβ1-4GlcNAcβ1-4GlcNAc, a series of oligomannose type oligosaccharides, $Man_{5-9}GlcNAc_2$ (about 8.5% of the total oligosaccharides),

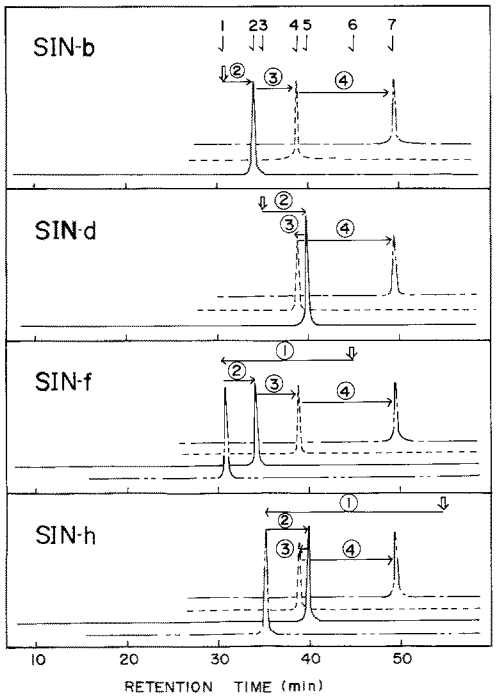


Figure 7. HPLC analysis of the products obtained from ABEE-oligosaccharides in SIN—b, d, f and h by sequential glycosidase digestion. ABEE-oligosaccharides in SIN—b, d, f and h were sequentially digested with: ①, bovine kidney α-fucosidase; ②, jack bean β-galactosidase; ③, jack bean β-*N*-acetylhexosaminidase; and ④, jack bean α-mannosidase. The products were analysed by ODS/HPLC under the conditions depicted in Fig. 2. Arrows (↓) indicate the elution positions of standard ABEE-oligosaccharides: 1, $G_2 \cdot GN_2 \cdot M_3 \cdot GN \cdot GN$-ABEE; 2, $GN_2 \cdot M_3 \cdot GN \cdot GN$-ABEE; 3, $G_2 \cdot GN_3 \cdot M_3 \cdot GN \cdot GN$-ABEE (obtained from NC—a by α-fucosidase digestion); 4, $M_3 \cdot GN \cdot GN$-ABEE; 5, $GN_3 \cdot M_3 \cdot GN \cdot GN$-ABEE (obtained from NC—c by α-fucosidase digestion); 6, $G_2 \cdot GN_2 \cdot M_3 \cdot GN \cdot F \cdot GN$-ABEE; 7, $M \cdot GN \cdot GN$-ABEE. Open arrows (↓) indicate the elution positions of intact ABEE-oligosaccharides.

and biantennary complex type oligosaccharides (about 64.3% of the total) with the core structures of Manα1-6 (±GlcNAcβ1 - 4)(Manα1 - 3)Manβ1 - 4GlcNAcβ1 - 4 (±Fucα1-6)GlcNAc. The glucose-containing oligosaccharides with 7 and 8 mannose residues have not previously been reported as a component of a mature glycoprotein.

Occurrence of the unusual monoglucosylated oligomannose type oligosaccharides with 7 and 8 mannose residues in addition to $Glc_1Man_9GlcNAc_2$ on a mature glycoprotein, IgY, poses interesting questions as to how these oligosaccharides are synthesized. Glucosylated oligomannose type oligosaccharides are known to be the key intermediates in the biosynthesis and processing of Asn-linked oligosaccharides. In the common processing pathway, soon after transfer of the lipid-linked $Glc_3Man_9GlcNAc_2$ to protein, the three glucose residues are removed in the endoplasmic reticulum, and then trimming of the α(1-2)-linked mannose residues occurs in the Golgi apparatus [25]. Godelaine *et al.* [26] have reported the formation of protein-linked $Glc_1Man_{7-9}GlcNAc_2$ in the endoplasmic reticulum of thyroid cells by pulse-chase studies, suggesting the possibility of alternative processing routes. They assumed that these oligosaccharides had been formed by deglucosylation and demannosylation of $Glc_3Man_9GlcNAc_2$. Trombetta *et al.* [27] have recently reported alternative pathways of oligosaccharide processing which directly transfer a glucose residue from UDP-Glc to $Man_{7-9}GlcNAc_2$ in glycoprotein and occur in the endoplasmic reticulum of various species such as mammalian, plant, fungal and protozoan cells. It remains to be clarified whether the monoglucosylated oligosaccharides in IgY are formed by the alternative pathways of oligosaccharide processing or by trimming of α(1-2)-linked mannose residues from $Glc_1Man_9GlcNAc_2$.

Human immunoglobulins, IgD [28], IgM [29] and IgE [30] contain both oligomannose and complex type oligosaccharides, whereas IgG [4–6] and IgA [31] contain only complex type oligosaccharides. It is very interesting that hen egg-yolk immunoglobulin, IgY, contained both oligomannose type and complex type oligosaccharides. Human IgG is composed of only complex type oligosaccharides: 4 classes of biantennary complex type oligosaccharides with or without fucose and bisecting *N*-acetylglucosamine, and biantennary oligosaccharides with fucose and without bisecting *N*-acetylglucosamine are the most predominant components [4–6]. In contrast, IgY contained biantennary oligosaccharides with fucose and bisecting *N*-acetylglucosamine as the major components.

Serum IgG has been shown to be transferred preferentially from blood circulation of the hen to the egg-yolk as the means of maternal transfer of antibody to chicks. Because this transfer is analogous to placental transfer in mammals, it will be important to compare the structures of sugar chains of serum IgG and egg-yolk IgY. Structural analysis of serum IgG is now in progress in our laboratory.

We have reported previously [12, 13] that derivatization of oligosaccharides with ABEE is very useful for separation and sensitive detection on HPLC and gel permeation chromatography. In the present study, we have found important characteristics of ABEE-oligosaccharides with ODS/HPLC. ABEE derivatives of oligosaccharides containing fucose linked to *N*-acetylglucosamine at the reducing end via an α(1-6) linkage were strongly retarded, whereas those containing bisecting *N*-acetylglucosamine were slightly retarded on the column. Thus, grouping the complex type oligosaccharides into biantennary without fucose and bisecting *N*-acetylglucosamine, biantennary with bisecting *N*-acetylglucosamine and without fucose, biantennary with fucose and without bisecting *N*-acetylglucosamine, and biantennary with fucose and bisecting *N*-acetylglucosamine can be achieved by ODS/HPLC. Within the group, the larger oligosaccharides were eluted earlier. The combination of Amide-80 HPLC, which can separate ABEE-oligosaccharides mainly based on their molecular sizes, and ODS/HPLC is useful for the separation and identification of Asn-linked

oligosaccharides. We are now developing a method for the two dimensional mapping of Asn-linked oligosaccharides by these HPLC systems.

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