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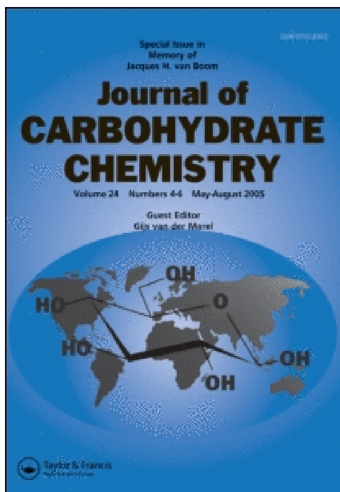

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An Efficient Preparation and Structural Characterization of Sialylglycopeptides from Protease Treated Egg Yolk

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AN EFFICIENT PREPARATION AND STRUCTURAL CHARACTERIZATION OF SIALYLGLYCOPEPTIDES FROM PROTEASE TREATED EGG YOLK

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ABSTRACT

Delipidated egg yolk (DEY) was digested with protease. The digest was ultrafiltered using a molecular weight cut-off of 10,000, and the UF permeate was treated with a reverse osmosis membrane. The resulting sialylglycopeptides-rich fraction was purified by the combination of anion exchange and gel filtration chromatography. Two major sialylglycopeptides, A-I and A-II, were characterized as biantennary complex type sialylglycopeptides having one or two *N*-acetylneuraminic acid and 1 - 3.5 amino acids including one asparagine residue by composition analyses and ^{1}H NMR spectroscopy.

INTRODUCTION

The use of carbohydrate based drugs and natural foods are becoming more widespread as more is known about the functions of sialic acid, sialyloligosaccharides[1] and their conjugates.[2] One must know the detailed molecular structures of such molecules to understand their interactions in the body. As examples, sialylglycoconjugates on the cell surface are known to serve as receptors for bacterial and viral infections. It has been suggested

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that sialic acids derivatives might be useful as competitive inhibitors against microbial infections. Itzstein et al.[3,4] have reported the 4-guanidino derivative of *N*-acetylneuraminic acid (NeuAc) as a novel inhibitor of influenza virus sialidase. Sialyloligosaccharides such as sialyl-Le^{X} and sialyl-Le^{a} have been characterized as carbohydrate ligands of inflammatory response[5] and cancer metastasis.[6,7]

Egg yolk is not only a nutritionally balanced food but also an excellent source of many chemical storehouses. In our continuing efforts to elucidate the biological roles of sialylglycoconjugates in hen's egg, we have reported the large scale preparation of *N*-acetylneuraminic acid from chalaza, egg yolk membrane[8] and delipidated egg yolk (DEY).[9] One type of sialic acid, NeuAc, has been found in hen's egg[8,10] in contrast to bovine submandibular gland in which 14 different sialic acids have been detected. In a previous paper by us,[11] we characterized the carbohydrate structures of sialyloligosaccharides prepared from the water-soluble fraction of delipidated egg yolk. The investigation of the biological roles of egg yolk sialyloligosaccharides necessitates the large scale preparation of sialyloligosaccharides.

In this study, we used whole DEY and established an efficient procedure for the preparation of sialylglycopeptides from the protease digested egg yolk and determined their structures, including the peptide moieties.

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RESULTS AND DISCUSSION

The sialylglycopeptides-rich fraction was obtained from delipidated egg yolk (DEY) by the treatment with protease as described in the "Experimental" section. Sialylglycopeptides-rich fraction (1.5 kg) was obtained from 40 kg of DEY. The sialic acid content of the sialylglycopeptides-rich fraction was estimated as 2.2%. The sialylglycopeptides-rich fraction was loaded on a column of Dowex MSA-1 (H^+ form, 30 × 50 cm) to purify sialylglycopeptides. The fraction eluted with 0.05 M NaCl contained maximum amounts of sialylglycopeptides (Fig. 1). This fraction was further chromatographed, using a preparative HPLC fitted with a gel filtration column (GS-520P), to yield fraction A which clearly showed the presence of sialic acid. The molecular weight of fraction A was estimated as approximately 3,000 based on the elution position of standard sample. The fraction A was finally purified to yield A-I and A-II fractions on DEAE-

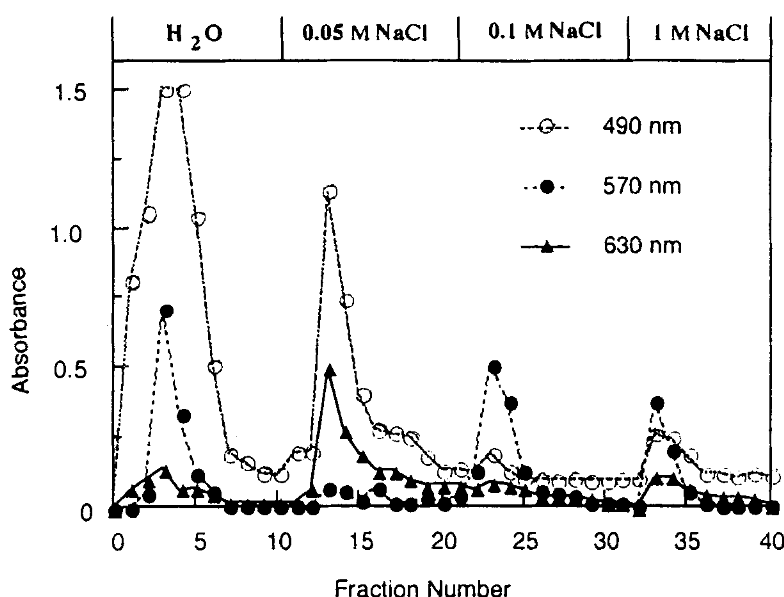

Figure 1. Elution profile of sialylglycopeptides-rich fraction on anion exchange column (Dowex MSA-1); sialic acid (-▲-); hexose (-O-); amino acids (-●-).

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TOYOPEARL 650M column (Tosoh Co., Ltd., Tokyo, Japan) (Fig. 2). The yield of A-I and A-II was estimated as 9.88 and 11.4 % of the sialylglycopeptides-rich fraction, respectively.

Based on the carbohydrate composition analysis the molar ratios of sialic acid of A-I and A-II were 0.82 and 1.7, respectively as shown Table 1. This result suggests that A-I and A-II might have the *N*-linked biantennary complex type mono- or di-sialyloligosaccharides, respectively. The amino acid composition analysis showed that the molar ratio of lysine (Lys) in A-II was 1.2 based on the molar ratio of asparagine (Asn) taken as 1.0. However, the molar ratios for the other amino acids in both A-I and A-II were less than 1.0 (Table 2), suggesting that A-I and A-II could have an average of 1 to 3.5 amino acids. A-I and A-II were further characterized by 400 MHz ^{1}H NMR. Four signals of H-1 for GlcNAc and chemical shifts of H-1 and H-2 for Man-4 and -4' indicated that A-I and A-II were biantennary complex-type oligosaccharides (Table 3).[11, 12, 13] In the spectrum of monosialyloligosaccharide A-I, the intensity of the signal at δ=1.720 ppm demonstrated the

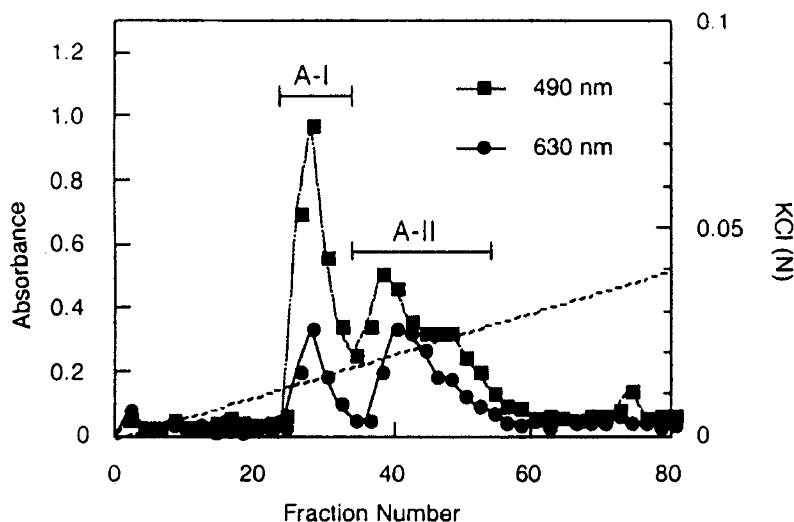

Figure 2. Elution profile of sialylglycopeptides, fraction A, on DEAE-TOYOPEARL 650M (2.5 × 50 cm) eluted with a linear gradient of KCl; sialic acid (-●-); hexose (-■-).

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Table 1. Carbohydrate Compositions of A-I and A-II Glycopeptides

	Man [a]	Gal	GlcNAc	Sia
A-I	3.0	1.9	3.7	0.82
A-II	3.0	1.9	3.5	1.7

a. Molar ratios of carbohydrates are relative to mannnose (Man) taken as 3.0.

Table 2. Amino Acid Compositions of A-I and A-II Glycopeptides

	Asx [a]	Ser	Glu	Gly	Ala	Val	Lys
A-I	1.0	0.19	0.20	0.13	0.32	0.36	ND [b]
A-II	1.0	0.19	0.63	0.18	0.12	0.20	1.2

a. Molar ratios of amino acids are relative to Asp taken as 1.0. b. not detected.

Table 3. ^{1}H NMR data (δ value) for the Monosaccharide Residues of A-I, A-II, and a Reference Glycopeptide.

```
 6'        5'        4'
NeuAcα2-6Galβ1-4GlcNAcβ1-2Manα1\
                                 \6                                  NH2
                                  Manβ1-4GlcNAcβ1-4GlcNAcβ1-Asn
                                 /3
NeuAcα2-6Galβ1-4GlcNAcβ1-2Manα1/   3         2           1        |
 6         5         4                                          COOH
                              Reference glycopeptide[12]         Lys
```

		A-I	A-II	Reference[12]
H-1 of	GlcNAc-1	5.050	5.051	5.088
	GlcNAc-2,5,5'	4.60~4.63	4.60~4.63	4.603, 4.616
	Man-4	5.137	5.137	5.133
	Man-4'	4.922	4.950	4.949
	Gal-6,6'	4.447, 4.474	4.447	4.442, 4.447
H-3ax of	NeuAc	1.720	1.719	1.716
H-3eq of	NeuAc	2.670	2.672	2.666, 2.672

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presence of a single NeuAc residue. The signal for H-1 of Gal (δ=4.474 ppm) was shifted downfield as compared with the signal of the reference glycopeptide. These results suggested that A-I had a single NeuAc having a linkage of α2→6 to galactose. The chemical shift values for βH of Asn (δ=2.7-2.9 ppm) and αH of Asn (δ=4.3-4.5 ppm) indicated that an Asn residue was linked to GlcNAc-1. The Lys residue was not observed in this spectrum. Therefore, the structure of A-I was proposed as in Fig. 3. In the spectrum of A-II, the chemical shifts for H-1 of GlcNAc-1, -2, -5 and 5' were similar to those obtained in A-I. The chemical shift for H-1 of Man-4' (δ=4.950 ppm) and for H-3ax and H-3eq of NeuAc (δ=1.719 and 2.672 ppm, respectively) indicated that two NeuAc residues were linked to Gal-6, -6' via α2→6 linkage. The chemical shift values for βH of Asn (δ=2.7-2.9 ppm), αH of Asn (δ=4.3-4.5 ppm) and εCH_2 of Lys (δ=3.01 ppm) indicated that Asn and Lys residues were linked to GlcNAc-1. The molar ratio of Lys residue was estimated as one mole from the intensity of εCH_2 of Lys. This result was consistent with the result of amino acid composition analysis. From these studies, the structure of A-II was concluded as shown in Fig. 3.

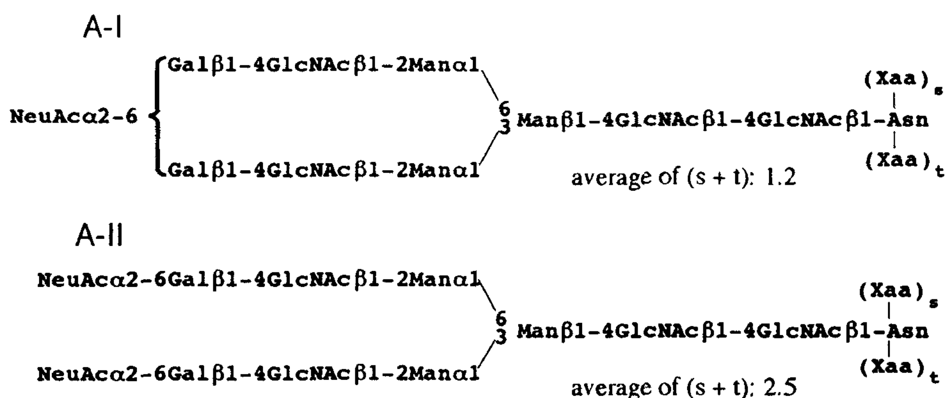

Figure 3. Structures of sialyloligosaccharides (A-I and A-II) form protease treated egg yolk.

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CONCLUSION

In this paper, we have established a procedure for an efficient preparation of sialylglycopeptides by the enzymatic digestion of egg glycoproteins. The structures were determined as the typical biantennary complex type sialylglycopeptides, which account for 21.3 % of total sialylglycopeptides of the sialylglycopeptides-rich fraction. The carbohydrate structures of sialylglycopeptides obtained by protease digestion of whole DEY were similar to the major sialyloligosaccharides of the water-soluble fraction of DEY which we previously reported.[11] The information on the complex structure of these materials obtained in this study will become useful for their application of medical foods and pharmaceuticals.

EXPERIMENTAL

Preparation of sialylglycopeptides-rich fraction. The delipidated egg yolk (DEY)[9, 11] (40 kg) was treated with 90 kU of protease (Orientase of *Aspergillus oryzae* origin, Hankyu Bioindustry Co., Ltd., Osaka, Japan) in 0.05 M phosphate buffer, pH 7.0 at 50 °C for 20 h. The enzyme reaction was monitored by the concentration of sialic acid.[14] The reaction mixture was filtered with the aid of Celite 500 (Celite Co., Ltd., Lompoc, CA, USA).

The filtrate from the enzyme digested fraction was passed through an ultrafiltration (UF) unit, which had a membrane of nominal molecular weight cut-off of 10,000, and a RUW-4 pump unit (Nitto Denko Co., Ltd., Tokyo, Japan). The UF permeate was dialyzed with a reverse osmosis (RO) membrane, NTR-7410 (Nitto Denko Co., Ltd.). Finally, the RO retentate was concentrated under reduced pressure at 45 °C to give the final sialylglycopeptides-rich fraction.

Fractionation and characterization of A-I and A-II. The sialylglycopeptides-rich fraction described above was chromatographed on a column of Dowex MSA-1 (H^+ form, 30 x 50 cm). Elution was performed stepwise with distilled water, 0.05 M NaCl, 0.1 M NaCl and 1 M NaCl, respectively at a flow rate of 100 mL/min. Sialic acid, hexose and amino acid were monitored by the resorcinol method,[15] phenol-H_2SO_4 method[16] and ninhydrin method.[17] HPLC of the fraction eluted 0.05 M NaCl (Figure 1) was performed with a LC-908 (Japan Analytical Industry Co., Ltd., Tokyo, Japan) using a GS-520P gel filtration column (2.15 × 50 cm, Asahi Chemical Industries Co., Ltd., Tokyo, Japan). The column was eluted isocratically with 5 % acetonitrile in 50 mM ammonium acetate at 3.0 mL/min. Elution was monitored by refractive index. The fraction A was further purified on DEAE-TOYOPEARL 650M column (1.5 × 50 cm). The column was eluted with a linear gradient of KCl of 0 - 0.04 N at a flow rate of 2 mL/min.

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Carbohydrate composition. A-I (50 μg) and A-II (50 μg) were methanolyzed with 0.5 N HCl-Methanol at 65 °C for 16 h, followed by *N*-reacetylation and trimethylsilylation (TMS) with hexamethyldisilazane and trimethylchlorosilane. The carbohydrate composition of the TMS-derivatives was determined by GLC (Shimadzu GC-9A gas chromatography) equipped with a glass column (1 m × 3 mm) packed with 1.5 % OV-17 on Chromosorb W at temperatures programmed from 160 to 250 °C (4 °C /min).[18,19] Molar ratios of carbohydrates are relative to Man taken as 3.0.

Amino acid composition. Amino acid composition was determined according to Moore and Stein.[20] A-I (3 mg) and A-II (3 mg) were hydrolyzed with 1 mL of 6M HCl at 110 °C for 24 h in sealed, evacuated tubes, respectively. The analyses were carried out on the resulting hydrolysate with an amino acid analyzer (Hitachi 835-50; Hitachi Co., Ltd., Tokyo, Japan).

^1H NMR spectrometry. Sialylglycopeptides were prepared by a three-fold lyophilization of the solution, finally using 99.96 % deutrated D_2O (Aldrich Inc., WI, USA)

as solvent. ^{1}H NMR spectra were recorded at 400 MHz with a JEOL-GSX-400 spectrometer (JEOL Co., Ltd., Tokyo, Japan), operating in the pulsed Fourier transform mode. The chemical shifts (δ) are expressed in ppm downfield from external sodium 2,2-dimethyl-2-silapentane-5-sulfonate (DSS), but were actually measured by reference to internal acetone (δ = 2.225 ppm).

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