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Note

The expression of sialylated high-antennary *N*-glycans in edible bird's nest

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Abstract—Edible bird's nest (EBN) is the nest made from the saliva of *Collocalia* swift. Recently, we have found that EBN extract could strongly inhibit infection of influenza viruses in a host-range-independent manner [Guo, C. T.; Takahashi, T.; Bukawa, W.; Takahashi, N.; Yagi, H.; Kato, K.; Hidari, K. I.; Miyamoto, D.; Suzuki, T.; Suzuki, Y. *Antiviral Res.* **2006**, *70*, 140–146]. Although this antiviral activity might be attributed to *O*- or *N*-glycoconjugates, no *N*-glycan structures have so far been described for EBN. Here, we report the N-glycosylation profile of EBN, in which a tri-antennary *N*-glycan bearing the α2,3-*N*-acetylneuraminic acid residues is displayed as a major component. We suggest that the sialylated high-antennary *N*-glycans of EBN contribute to the inhibition of influenza viral infection.

Keywords: Edible bird's nest; *Genus collocalia*; Sialic acid; *N*-Glycan; HPLC mapping

Edible bird's nest (EBN) is the dried form of the nest of a male *Collocalia* swift that is made from its saliva. Recently, we have found that an EBN extract could neutralize infection of influenza viruses in MDCK cells and inhibit the hemagglutination of human erythrocytes by influenza A viruses originating from various hosts, for example, human, avian, and porcine.[1] This antiviral activity has been suggested to be attributed to some sialylglycoconjugates contained in EBN. Although the structural data of *O*-glycans[2] and glycosaminoglycans[3] have been reported for EBN, no *N*-glycan structures have so far been described for this substance. In this paper, we characterize the structures of *N*-glycans derived from EBN by a combined use of multi-dimensional high-performance liquid chromatography (HPLC) and mass spectrometric (MS) methods.

N-Glycans from EBN were released by using glycoamidase A, labeled with 2-aminopyridine, and

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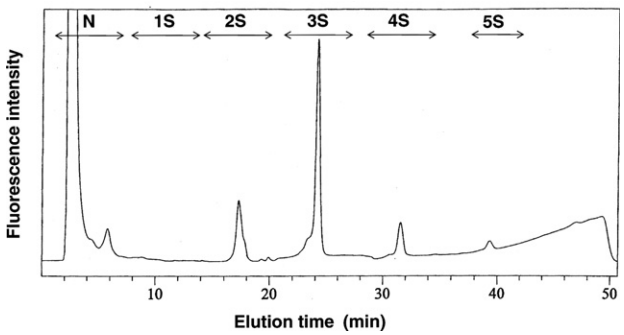


Figure 1. Elution profile of PA-*N*-glycans derived from EBN on a DEAE column. The PA-glycan mixture was separated according to sialic acid contents. N, neutral; 1S, mono-sialyl; 2S, di-sialyl; 3S, tri-sialyl; 4S, tetra-sialyl; 5S, penta-sialyl.

subsequently subjected to multi-dimensional HPLC profiling.[4–6] Figure 1 shows the N-glycosylation profile on a diethylaminoethyl (DEAE) anion-exchange column of the pyridylaminated (PA)-glycans, which were separated according to the degrees of sialylation. The di-, tri-, tetra-, and penta-sialyl fractions were further separated on an octadecyl silica (ODS) column (Fig. 2). While, the neutral and mono-sialyl fractions were not detected the PA-glycans in the elution profiles on the ODS column (data not shown). The individual fractions separated on the ODS column were further applied onto an amide-silica column to record the elution time data on the two kinds of columns. The PA-oligosaccharides were identified on the basis of coincidence of the elution time normalized in GU with those in the GALAXY database.[7] For example, the major *N*-glycan corresponding to peak 3S-2 was eluted at 20.3 GU on the ODS column and at 7.8 GU on the amide column. The elution dataset is in good agreement with that of a known reference *N*-glycan, Neu5Ac-α-(2→3)-Gal-β-(1→4)-GlcNAc-β-(1→2)-Man-α-(1→6)-[Neu5Ac-α-(2→3)-Gal-β-(1→4)-GlcNAc-β-(1→4)-[Neu5Ac-α-(2→3)-Gal-β-(1→4)-GlcNAc-β-(1→2)-]Man-α-(1→3)-]Man-β-(1→4)-GlcNAc-β-(1→4)-[Fuc-α-(1→6)-]GlcNAc-PA (code no. 3A4-310.8 in GALAXY). Using co-chromatography and the Matrix-assisted laser desorption/ionisation time-of-flight (MALDI-TOF)-MS analyses, the structure of this PA-oligosaccharide was confirmed. PA-glycans corresponding to fractions 2S-1, 2S-2, 2S-3, 4S-2, and 5S-1 did not agree with any of the PA-glycans so far registered in GALAXY. These PA-glycans were trimmed by exoglycosidase treatments[8] to become identical to known ones. Taking into account the specificities of the exoglycosidases used, the original structures of these PA-glycans were uniquely determined. Thus, we identified all *N*-glycans derived from the EBN, except for fraction 4S-2.

MALDI-TOF-MS data indicated that the PA-glycans corresponding to fraction 4S-2 were the isomer of *N*-glycan 4S-1. Terminal Fuc, 3-linked Gal, and 4-linked GlcNAc were detected in gas chromatography (GC)–MS linkage analysis of the PA-glycan corresponding to peak 4S-2 as expected, along with 2,4- and 3,6-linked Man (Table 1). The total ion chromatogram (TIC) response indicated that the amount of 2,4-linked Man is about twice as much as that of 3,6-linked Man. Other doubly- or triply-linked mannose (e.g., 2,6-, 4,6-, or 3,4,6-linked Man) were not detected at a comparable level. The *N*-glycan 4S-2 was subjected to α2,3-sialidase and thereby released four Neu5Ac per molecule. The resultant desi-

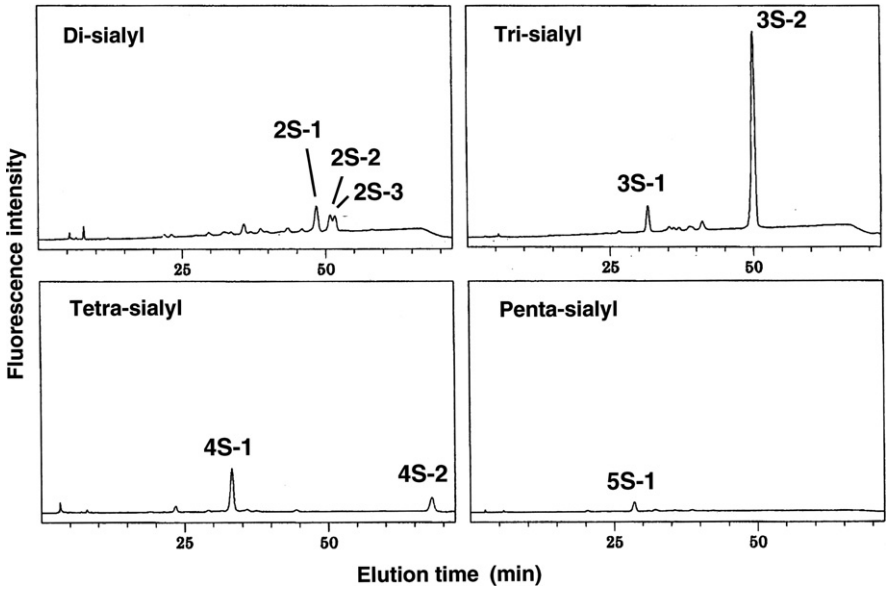


Figure 2. N-Glycosylation profiles on an ODS column of di-, tri-, tetra-, and penta-sialyl fractions.

Table 1. GC–MS linkage analysis of partially methylated alditol acetates prepared from the PA-glycan 4S-2

Elution time (min)	Characteristic fragment ions	Assignment[a]
14.29	118, 131, 162, 175	Terminal fucose
17.24	118, 129, 161, 234	3-Linked galactose
18.08	130, 190, 233	2,4-Linked mannose
18.68	118, 129, 189, 234	3,6-Linked mannose
20.43	117, 159, 233	4-Linked *N*-acetyl-glucosamine

[a] The respective partially methylated alditol acetates were identified by elution time and their characteristic fragment ions afforded by standard EI-MS analysis, in comparison to authentic standards.

alylated glycan was made able to release four Gal residues upon β-galactosidase treatment. Collision-induced dissociation (CID)-MS/MS analysis of this degalactosylated product indicated that both 1,6Man and 1,3Man arms possessed two GlcNAc residues (Fig. 3). All these data indicate that the tetra-sialyl glycan corresponding to 4S-2 is Neu5Ac-α-(2→3)-Gal-β-(1→4)-GlcNAc-β-(1→4)-[Neu5Ac-α-(2→3)-Gal-β-(1→4)-GlcNAc-β-(1→2)-]Man-α-(1→6)-[Neu5Ac-α-(2→3)-Gal-β-(1→4)-GlcNAc-β-(1→4)-[Neu5Ac-α-(2→3)-Gal-β-(1→4)-GlcNAc-β-(1→2)-]Man-α-(1→3)-]Man-β-(1→4)-GlcNAc-β-(1→4)-[Fuc-α-(1→6)-]GlcNAc-PA.

Table 2 summarizes the structures of the identified *N*-glycans and their incidence in EBN. Intriguingly, EBN expressed highly branched *N*-glycan structures possessing α2,3-NeuAc residues. These *N*-glycan structures were consistent with our previous data based on lectin blotting and monosaccharide analyses.[1] In addition, this is the first description of the multi-antennary sialylated *N*-glycan structures corresponding to peak 4S-2 and 5S-1. The atypical GlcNAc linkage, that is, GlcNAc-β-(1→4)-Man-α-(1→6)-Man, has so far been reported for fish,[9] pigeon,[8] and hen.[10,11]

It has been demonstrated that sialic acid clusters[12] such as mucin expressing sialylated *O*-glycans can multi-valently interact with viral hemagglutinin, and thereby, exhibit inhibitory activity against influenza virus.[13–15] On the basis of the present N-glycosylation profiling data, we suggest that the sialylated high-antennary *N*-glycans contribute to the inhibition against influenza viral infection of EBN, which can therefore be a safe and valid natural source for the prevention of influenza.

1. Experimental

1.1. Materials

Materials used for the experiments were purchased from various sources. Glycoamidase A from sweet almond, β-galactosidase from jack bean, and β-*N*-acetylhexosaminidase also from jack bean were purchased from Seikagaku Kogyo Co. (Tokyo, Japan). α-Sialidase from *Arthrobacter ureafaciens* was from Nacalai Tesque (Kyoto, Japan). α2,3-Sialidase from *Salmonella typhimurium* was from TAKARA Bio Inc. (Otsu, Japan). Trypsin and chymotrypsin were from Sigma Chemical Co. (St. Louis, MO, USA). Finally, the PA-derivatives of isomalto-oligosaccharides (degree of polymerization of glucose residues) and the PA-derivatives of neutral N-linked oligosaccharides of code nos. 300.5, 300.8, 310.8, and 410.16 were purchased from Seikagaku Kogyo Co.

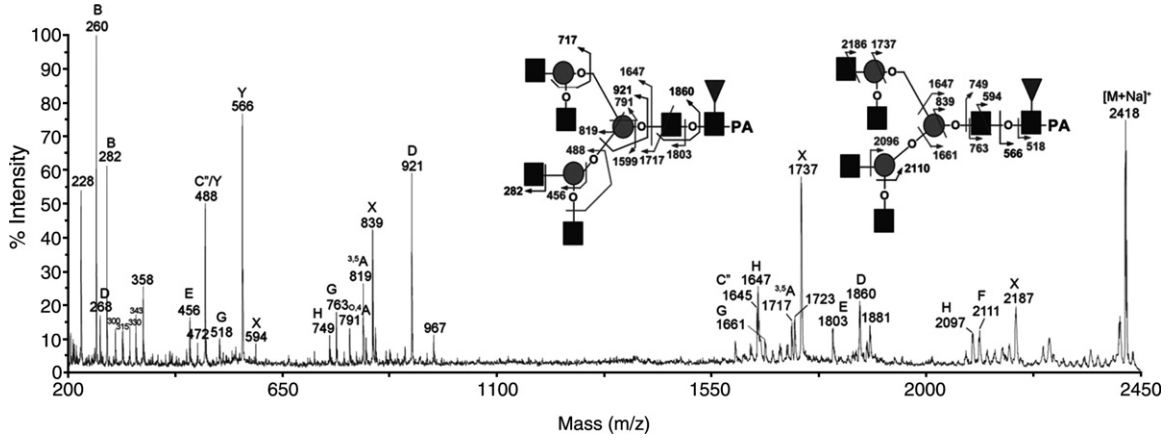
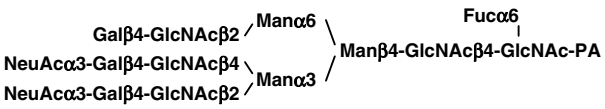
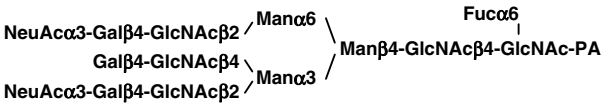
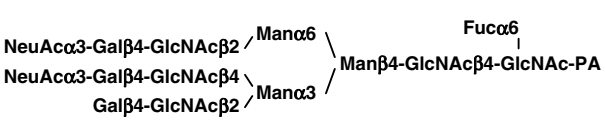
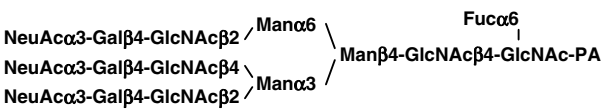
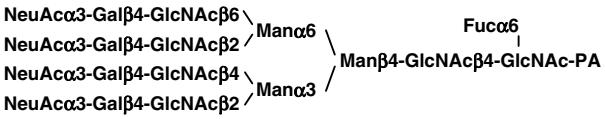
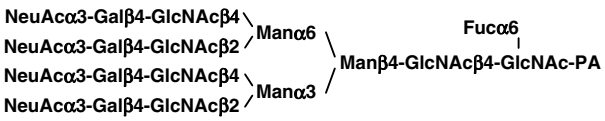
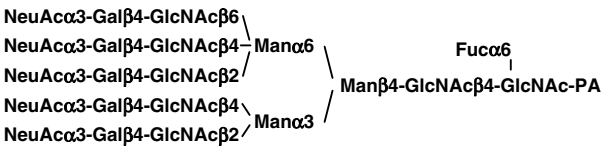


Figure 3. MS/MS sequencing of desialyl and degalactosyl PA-glycan corresponding to fraction 4S-2. The PA-tagged sample was permethylated after removal of sialic acids and β-galactoses. Y ion at *m/z* 566 indicated that it is a core fucosylated structure and that the Fuc is not located elsewhere. The tetra-antennary structure was demonstrated by the X ion series at *m/z* 2187, 1737, 839 coupled with B ion at *m/z* 282. No lacdiNAc or other terminal epitope detected apart from the single GlcNAc. No specific ion indicative of bisecting GlcNAc could be detected. ●, mannose; ■, *N*-acetylglucosamine; ▲, fucose.

Table 2. *N*-Glycans derived from EBN

Peaks (code no.[a])	Structures	Glucose unit		Relative quantity (%)
		ODS	Amide	
2S-1 (2A6-310.8)		19.7	7.8	5.7
2S-2 (2A4-310.8)		20.7	7.8	4.4
2S-3 (2A5-310.8)		21.0	8.0	3.8
3S-1	Epimer of 3S-2[b]	14.0	7.8	6.0
3S-2 (3A4-310.8)		20.3	7.8	57.0
4S-1 (4A2-410.16)		14.7	8.8	11.6
4S-2 (4A2-410.18)		28.1	8.5	4.9
5S-1 (5A2-510.51)		13.7	9.5	2.3

[a] The PA-oligosaccharides are coded according to the literature.[7]
[b] PA-glycan 3S-1 is the epimeric by-product of the pyridylamination reaction.

The EBN sample was obtained from a house nest derived from *Collocalia fuciphaga* in Indonesia as described previously.[1]

1.2. Methods

1.2.1. HPLC mapping method. All experimental procedures used, including the chromatographic conditions and glycosidase treatments, have been described previously.[4–6] The EBN extract, which was prepared by proteolysis with Pancreatin F,[1] was used as the starting material. The extract was further proteolyzed with chymotrypsin and trypsin mixture, and was further digested with glycoamidase A to release *N*-glycans. After the removal of the peptide materials, the reducing ends of the *N*-glycans were derivatized with 2-aminopyridine (Wako, Osaka, Japan). The mixture of PA-derivatives of the *N*-glycans was separated by HPLC on a TSK-gel DEAE-5PW column (Tosoh, Tokyo, Japan) according to their sialic acid content. Then, the PA-*N*-glycans were individually separated and identified sequentially on a Shim-pack HRC-ODS column (Shimadzu, Kyoto, Japan) and on a TSK-gel Amide-80 column (Tosoh, Tokyo, Japan). The identification of *N*-glycan structures were based on their elution positions on these three kinds of columns in comparison with PA-glycans in the GALAXY database.[7] On the other side, the structures of PA-glycans so far not registered in GALAXY were characterized by exoglycosidase treatments and mass spectrometric techniques as described previously.[5,6,16]

1.2.2. MALDI-MS/MS sequencing and gas chromatography–electron impact MS (GC–EI-MS) methylation analysis. The glycans were permethylated using the NaOH/dimethyl sulfoxide slurry method as described by Dell et al.[17] MALDI-MS and MS/MS analyses of

permethylated glycans were performed on a 4700 Proteomics Analyzer (Applied Biosystems, Framingham, MA), operated in the reflectron mode. For MS acquisition, the samples dissolved in acetonitrile were mixed 1:1 with 2,5-dihydroxybenzoic acid (DHB) matrix (10 mg/mL in water) and spotted on the target plate. For CID-MS/MS sequencing, the potential difference between the source acceleration voltage and the collision cell was set at 3 kV to obtain the desirable high energy CID fragmentation pattern. The indicated collision cell pressure was normally increased from 3.0×10^{-8} torr (no collision gas) to 5.0×10^{-7} torr (argon). The other processes were exactly conducted as described before.[18]

For GC–EI-MS linkage analysis, the partially methylated alditol acetates were prepared from permethyl derivatives by hydrolysis (2 M trifluoroacetic acid, 120 °C, 2 h), reduction (10 mg/mL $NaBD_4$, 25 °C, 2 h), and acetylation (Ac_2O, 100 °C, 1 h). GC–EI-MS was carried out using a Hewlett–Packard Gas Chromatograph 6890 connected to an HP 5973 Mass Selective Detector. The sample was dissolved in hexane before the splitless injection into an HP-5MS fused silica capillary column (30 m × 0.25 mm internal diameter, Hewlett–Packard). The column head pressure was maintained at ≈56.6 kPa to give a constant flow rate of 1 mL/min using helium as carrier gas. The initial oven temperature was held at 60 °C for 1 min, increased to 90 °C in 1 min, and then to 290 °C in 25 min at a rate of 8 °C/min.

Acknowledgments

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