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Distribution of *N*-acetylneuraminic acid and sialylglycan in eggs of the Silky fowl

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Abstract 1. The distribution of sialic acid in the eggs of original Silky fowl was investigated. The sialic acid contents of the yolk, albumen and the chalaza of a single egg were 205·2, 11·96 and 0·83 mg, respectively.
2. The sialic acid content of the yolk of Silky eggs was 11·5-fold higher than that of a conventional domestic fowl yolk.
3. Sialic acid isolated from Silky yolk was entirely *N*-acetylneuraminic acid (NeuAc). No *N*-glycolylneuraminic acid or *O*-acetyl containing sialic acid was observed.
4. The structure of the major sialylglycan in Silky egg yolk was determined to be a disialyl-biantennary chain in which the NeuAc residues were α2–6 linked to glucose. No α2–3 linkage was observed.
5. Thus, the Silky fowl's egg provides an excellent source of NeuAc and sialylglycan.

INTRODUCTION

The original Silky fowl is mild and short, with a small and long head but a short neck. It can be easily distinguished from other chickens. The egg of the original Silky fowl is well known in the Orient and for thousands of years has been credited with medicinal and health-promoting values. However, a modern scientific approach has only recently been applied to determine its medicinal chemical and biochemical components (Ferrand and L'Hermite, 1985; Sakakibara *et al*., 2000). Recently, the *N*-acetylneuraminic acid (NeuAc) and sialyloligosaccharide derivatives present in hen's eggs have been extensively studied (Juneja *et al*., 1991; Koketsu *et al*., 1992, 1993; Koketsu, 1997; Seko *et al*., 1997). Sialic acid is important in cell–cell interactions. Sialylglycoconjugates reportedly exhibit many biological functions, such as receptors for rotavirus (Willoughby and Yolken, 1990; Willoughby, 1993; Koketsu *et al*., 1995, 1997), *Vibrio cholerae* (Schengrund and Ringler, 1989; Masserini *et al*., 1992) and for somatostatin in rat brain AtT-20 cells (Rens-Domiano and Reisine, 1991). Sialylglycoconjugates are also known to modulate cell–cell interactions and viscosity, and determine negative charge on the surface of cells (Schauer, 1982). While sialic acid can be taken up by eukaryotic cells (Oetke *et al*., 2001), its primary origin in animals is through biosynthesis (Jacobs *et al*., 2001). Although sialic acid and sialylglycoconjugates have been prepared from animal tissues (Wang *et al*., 2001), hen's eggs and by synthesis (Maru *et al*., 1998), there are no reports of sialic acid from the egg of the Silky fowl. The object of the current study was to clarify distribution of sialic acid in eggs of Silky fowl and to determine the structure of its sialyloligosaccharide.

MATERIALS AND METHODS

Materials

Authentic NeuAc of *Escherichia coli* origin was purchased from Nakarai Tesque Inc., Tokyo, Japan. Eggs of Silky fowl were a kind gift from Tokai Biken Co., Ltd, Gifu, Japan. Each fresh egg fraction was obtained from the eggs collected within half a day after laying by a Silky fowl, and immediately used as starting material for these experiments.

Colorimetric analysis of sialic acid

The sialic acid was quantified by the modified periodate thiobarbituric acid (TBA) method (Warren, 1959). Sialic acid, liberated from sialylglycoconjugates in each fraction by heating the materials in 0·05 M sulphuric acid at 80°C, was colorimetrically determined. The content of sialic

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acid in each fraction was calculated from the means of data, from 10 different eggs.

Preparation of the sialic acid from egg yolk of Silky fowl

The egg yolk of Silky fowl was homogenised with three volumes of ethanol and the resulting suspension was filtered. The residue was homogenised with three volumes of water. The resulting suspension was acidified to pH 1·4 with 6 M hydrochloric acid and heated for one hour at 80°C. After cooling, 6 M sodium hydroxide was added until the desired pH of 6·0 was attained. The material was applied to a Dowex HCR-W2 column (1·5 × 30 cm, H^+ form, 20 to 50 mesh), followed by a Dowex 1 × 8 (1·5 × 30 cm, formate form, 200 to 400 mesh) column. The second Dowex column was washed with water and eluted with a 0 to 2 N linear gradient of formic acid at a flow rate of 2 ml/min. The sialic acid fractions were collected and evaporated at 45°C under reduced pressure. The residue was decolourised with activated charcoal and then lyophilised.

400 MHz NMR analyses

NMR spectra were measured in deuterium oxide (D_2O) by a JEOL-α-400 instrument (JEOL Co., Ltd, Tokyo, Japan) operated in the pulsed Fourier-transform mode. Sodium 4,4-dimethyl-4–siapentanesulfonate was used as an internal standard.

Isolation of sialylglycopeptide from egg yolk of Silky fowl

The isolation of sialylglycopeptide was carried out according to previously described procedures (Seko *et al.*, 1997). All the procedures were performed at 4°C. Fresh yolk (40 ml) obtained from three unfertilised eggs was added to an equal volume of water. The diluted egg yolk was immediately mixed with 1/10 volume of phenol/water (9:1, w/w) and the mixture was vigorously stirred for 30 min at 0°C. Water (100 ml) was added to the resulting emulsion and the mixture was centrifuged at 3400 rpm for 30 min. The supernatant was concentrated under reduced pressure and after removing insoluble material, applied to a Sephadex G-50 gel filtration column (2·1 × 109 cm, equilibrated and eluted with 0·1 M sodium chloride). The elution was monitored by the resorcinol–hydrochloric acid method for the determination of sialic acid (Jourdian *et al.*, 1971) and the phenol–sulphuric acid method for the determination of hexoses (Hodge and Hofreiter, 1962). The sialic acid-positive fractions were collected and re-chromatographed on the same column. After desalting through a Sephadex G-25 column (2·3 × 68 cm, equilibrated and eluted with 5% aqueous ethanol), the effluent was applied on a DEAE-Toyopearl 650 M anion exchange column (Tosoh Co., Ltd, Tokyo, Japan, 2·5 × 16 cm, Cl^- form, equilibrated with 5 mM Tris–hydrochloride buffer at pH 8·0). The adsorbed material was eluted by a 0 to 0·1 M sodium chloride linear gradient in the same buffer. The sialylglycopeptide eluted in the flow-through of the column. The fractions corresponding to the sialylglycopeptide were combined and further purified by cation exchange chromatography on a CM-Sephadex C-25 (2·3 × 68 cm, Na^+ form, equilibrated with 10 mM sodium acetate buffer pH 5·5) before desalting and lyophilisation.

PNGase F digestion of sialylglycopeptide

PNGase F digestion was performed according to the method of Tarentino and Plummer (1987). Sialylglycopeptide (6 mg), dissolved in 0·6 ml of 0·25 M sodium phosphate (pH 8·6) containing 4 U of PNGase F (Boehringer Mannheim, Germany), was incubated at 37 °C for 16 h under a drop of toluene. The free glycan fraction was desalted by Sephadex G-25 gel filtration (1·4 × 70 cm, equilibrated and eluted with 5% aqueous ethanol) and then subjected to structural analyses using labelling.

Labelling of oligosaccharides with *p*–aminobenzoic ethyl ester (ABEE) and analysis of the resulting derivatives by HPLC

ABEE labelling and analysis of the resulting derivatives were performed according to previously reported methods (Koketsu *et al.*, 1993). The columns used were TSKgel DEAE–5PW (0·75 × 7·5 cm, Tosoh Co., Ltd) and Wakosil 5C18-200. In the case of the TSKgel DEAE–5PW column, the ABEE derivatives were eluted with a 10 to 250 mM linear gradient of monobasic sodium phosphate at a flow rate of 1·0 ml/min at 30°C. In the case of the Wakosil 5C18-200 column, elution was done with 8% aqueous acetonitrile containing 50 mM monobasic sodium phosphate at a flow rate of 1·0 ml/min at 25°C. The eluate was monitored at 304 nm.

RESULTS AND DISCUSSION

The sialic acid content in the yolk, albumen and chalaza of the egg of Silky fowl was measured by the TBA colorimetric method. Sialic acid was distributed in all three fractions of the egg. The yolk fraction contained the most significant proportion of sialic acid (205·2 mg/whole egg, 0·71% in yolk). When compared with results on the sialic acid content of conventional hen's egg,

Table 1. *Distribution of N-acetylneuraminic acid per egg of Silky fowl and conventional hen*

Fraction	Silky fowl egg			Hen egg[1]		
	Quantity (g)	NeuAc		Quantity (g)	NeuAc	
		(mg)	(%)		(mg)	(%)
Yolk	28·9	205·2	0·71 ± 0·099	18·7	17·77	0·095
Albumen	24·4	11·96	0·049 ± 0·040	40·5	4·05	0·01
Chalaza	0·553	0·83	0·15 ± 0·064	0·0148	0·27	1·8
Total	53·85	218·0	0·40 ± 0·058	59·21	22·09	0·037

[1]Juneja *et al.* (1991).

reported previously (Juneja *et al.*, 1991), the sialic acid content of Silky yolk was 11·5-fold higher (Table 1).

Pure sialic acid was isolated from delipidated egg yolk of Silky fowl. Sialic acid (1·5 g) was purified from 300 g of the yolk by the procedure described in the Materials and Methods section. The ^{1}H and ^{13}C NMR spectra of the purified sialic acid were consistent with NeuAc. TLC and NMR spectra of the compound confirmed that there were no *N*-glycolyl or *O*-acetyl groups in the compound as in the results obtained on conventional hen egg yolk.

Fresh yolk from three eggs (40 ml) was deproteinised by phenol treatment using the method described in Materials and Methods and the supernatant was subjected to Sephadex G-50 gel chromatography. After desalting, the fractions were applied to a DEAE-Toyopearl 650 M anion exchange column. Both hexose- and sialic acid-positive fractions were recovered in the flow-through solution. The flow-through fractions were further purified using a CM-Sephadex C-25 cation exchanger. Almost all of the hexose and sialic acid were recovered as flow-through fractions. The yield of sialylglycopeptide was 9·3 mg from a single yolk. Incubation with PNGase F completely released the glycan moiety of sialylglycopeptide, which was purified by Sephadex G-50 gel chromatography. To characterise the isolated glycan, the reducing end of the glycan was labelled with ABEE and analysed by HPLC with a DEAE-5PW anion exchange column and a Wakosil 5C18-200 reversed phase column as described in Materials and Methods. Only a single peak resulted and it eluted from two chromatographic systems at the same retention times as the ABEE-labelled disialylbiantennary glycan chain, previously characterised as having a branch structure, NeuAcα2–6Galβ1–4GlcNAcβ1–2Man (data not shown). Therefore, it is concluded that sialylglycan has a homologous disialyl-biantennary glycan chain of the same structure.

The chemical shift values of the structural reporter groups of the ABEE-labelled sialylglycan are summarised in Table 2, with those of reference sialylglycan reported by Koketsu *et al.* (1993). The signals for H-3ax and H-3eq of NeuAc, 1·724 and 2·667 ppm, respectively, supported that the NeuAc residues of sialylglycan have an α2–6 linkage to glucose rather than an α2–3 linkage (H-3ax, 1·796 to 1·802 ppm; H-3eq, 2·756 to 2·758 ppm) (Vliegenthart *et al.*, 1983). The chemical shift values for the other saccharide residues of the sialylglycan closely resemble those of the reference sialylglycan, thereby strongly suggesting that sialylglycan has a glycan chain identical to that of the reference sialylglycan. From these results, the chemical structure of ABEE-labelled sialylglycan is proposed as shown in the Figure.

Table 2. *^{1}H nuclear magnetic resonance (NMR) chemical shifts for the structural reporter groups of ABEE-labelled sialylglycan from Silky fowl egg yolk and ABEE-labelled reference sialylglycan*

Reporter group		Sialylglycan	Reference[1]
H-1 of	GlcNAc-2	4·628	4·625
	GlcNAc-5,5′	4·608	4·6
	Man-3	4·774	ND[2]
	Man-4	5·139	5·132
	Man-4′	4·946	4·946
	Gal-6,6′	4·451	4·442
H-2 of	Man-3	4·254	4·240
	Man-4	4·200	4·193
	Man-4′	4·116	4·115
H-3ex of	NeuAc	1·724	1·718
H-3eq of	NeuAc	2·667	2·669

[1]Koketsu *et al.* (1993).
[2]Value could not be determined merely by inspection of the spectrum.

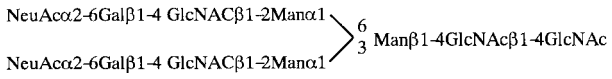

Figure. *Structure of major sialylglycan in Silky fowl egg yolk.*

In conclusion, the egg yolk of Silky fowl contains larger amount of NeuAc, 9·3-fold higher than the total NeuAc of a conventional hen's egg, and more sialylglycan than found in the hen's egg. Thus, Silky fowl egg yolks represent an excellent source of NeuAc and bi-antennary sialylglycan. Since the primary diet of fowl is plant-derived and plants do not contain sialic acid, it is likely that the differences in the sialic

acid content of Silky fowl and hen eggs results from differences in the extent of sialic acid biosynthesis in these animals.

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