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Preparation of *N*-acetylneuraminic acid from delipidated egg yolk*

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Egg yolk, a large proportion of the egg, was studied for the preparation of *N*-acetylneuraminic acid (Neu5Ac). The delipidated hen egg yolk (DEY; 500 kg containing 0.2% w/w, Neu5Ac) was hydrolysed with HCl (pH 1.4) at 80 °C and neutralized with NaOH (pH 6.0). The mixture was filtered and electrodialysed until the conductivity was 240 μS cm^{-1}. The filtrate was applied on a column of Dowex HCR-W2 (20–50 mesh), followed by a column of Dowex 1-X8 (200–400 mesh). The latter column was washed with water, and then eluted with a linear gradient of HCO_2H (0–2 M). The eluates containing Neu5Ac were concentrated using a reverse osmosis membrane and, finally, rotary evaporated at 40 °C. The residue was then lyophilized to yield 500 g Neu5Ac. The purity of Neu5Ac was >98% (TBA method). HPLC, NMR spectroscopy and TLC chromatography of the product obtained from the DEY showed that Neu5Ac was the sole derivative present in egg yolk. The DEY, a byproduct from egg processing plants, was found to be an excellent source for the large-scale preparation of Neu5Ac.

Keywords: sialic acid, *N*-acetylneuraminic acid, delipidation, egg yolk, electrodialysis.

Abbreviations: Neu5Ac, *N*-acetylneuraminic acid; Neu5Gc, *N*-glycolylneuraminic acid; DEY, delipidated egg yolk; HPLC, high performance liquid chromatography; TLC, thin layer chromatography; NMR, nuclear magnetic resonance; IR, infrared spectroscopy.

Sialic acid as a component of glycoconjugates (mainly glycoproteins and glycolipids) is present in biological materials. Recently, interest in sialic acid compounds has increased, as their role in many biological processes becomes known and further explored [1–4]. Many chemical and biotechnological companies are exploring the role of *N*-acetylneuraminic acid (Neu5Ac) and other sialic acids in cell function in order to create a new class of carbohydrate based drugs. Researchers from several groups have recently identified carbohydrate molecules that may lead to the development of effective oral anti-inflammatory drugs with minimum side effects [5, 6]. It has been found that cells carrying a carbohydrate ligand containing Neu5Ac (known as sialyl-Lewis X) were able to bind endothelial leukocyte adhesion molecule-1.

Sialic acids are considered to be important components for the protection of life. Sialic acids carry out various biological functions by their negative charge, by acting as receptors for microorganisms, toxins and hormones, by masking receptors and immunological recognition sites of molecules and cells [1]. Sialic acids have also gained increasing attention in pathological processes (e.g., sialidosis) [7, 8] and malignancies [9]. The determination of these sugars may be useful for the diagnosis and prognosis of cancer [9].

Therefore, the large scale preparation of Neu5Ac is desired. Neu5Ac has been mainly produced on a large scale from four major sources [10–12]. Measurement of the Neu5Ac contents in various fractions of hen egg (fresh egg) showed that Neu5Ac is distributed in all parts of egg, but is mainly located in chalaza, egg yolk membrane and egg yolk [10]. Previously, we reported the large scale preparation of the Neu5Ac from chalaza and egg yolk membranes of hen egg [10].

In the present investigation, delipidated egg yolk (DEY) powder, a byproduct of the egg yolk processing plant, was used as a raw material and a process flow was established for the large scale preparation of Neu5Ac.

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Materials and methods

Materials

Authentic Neu5Ac from *Escherichia coli* was purchased from Nakalai Tesque Inc., Tokyo, Japan [12]. Authentic *N*-glycolylneuraminic acid (Neu5Gc), from porcine submaxillary glands, was purchased from Sigma Chemical Co. (St. Louis, MO, USA).

Analysis of sialic acid

1. Colorimetric. The Neu5Ac was quantified by the modified periodate thiobarbituric acid (TBA) method [13]. Neu5Ac liberated by heating the materials in 0.05 M H_2SO_4 at 80 °C was monitored colorimetrically.

2. TLC method. TLC employed 1-propanol–1 M ammonium hydroxide–water, 6:2:1 by vol, as developing solvent with aluminium sheets of silica gel 60 (E. Merck, Germany); detection was achieved by spraying with 5% H_2SO_4 in MeOH and heating for 5 min at 150 °C.

3. HPLC method. HPLC analysis was performed with an EYELA PLC-5D (Tokyo Rikakikai Co. Ltd, Tokyo, Japan) attached to a Hitachi D-2500 integrator operated at 206 nm. The column used was Bio-Rad HPX-87H (Bio-Rad Laboratories, Tokyo, Japan) and the mobile phase (0.003 M H_2SO_4) flow was 0.65 ml min^{-1}, under a pressure of 57 kg cm^{-2} at ambient temperature. The sample (5 µl) was injected and run for 30 min.

4. NMR method. NMR spectra were obtained by ^2H_2O using a JEOL-GSX-400 instrument (JEOL Co. Ltd, Tokyo, Japan) operated in the pulsed Fourier transform method. Sodium 4,4-dimethyl-4-silapentanesulfonate was used as an internal standard.

Measurement and analysis of amino acids

1. Colorimetric method. The amino acids of the eluates obtained from the Dowex 1-X8 column were measured by the ninhydrin method [14].

2. Amino acid analyser. The amino acid composition of the hydrolysed DEY and the eluates of chromatography was determined as described by Moore and Stein [15]; the samples were hydrolysed with 6 M HCl at 110 °C for 80 h in sealed, evacuated tubes. The analyses were carried out on the resulting hydrolysate with an amino acid analyser (Hitachi 835-50; Hitachi Co. Ltd, Tokyo, Japan).

Electrodialysis of the hydrolysed DEY

The hydrolysed DEY, adjusted to the desired pH, was electrodialysed with TS-2-10 and TS-10-360 (Tokuyama Soda Co. Ltd, Tokyo, Japan) for the laboratory scale and large scale preparation of Neu5Ac, respectively. The electrodialysis system consisted of ion exchange membranes 'NEOSEPTA' CM-1 & AFN and CM-2 & ACS in the first step and second step, respectively. The system was a fully automatic batch type. A conductivity meter was included with the desalination vessel for the electrodialysis. The electrodialysis was carried out at room temperature (around 25 °C).

Preparation of the Neu5Ac from DEY

1. Laboratory scale. The DEY (2 kg) was homogenized with three volumes of water. The suspension was acidified to pH 1.4 with 6 M HCl and heated for 1 h at 80 °C. After cooling, 6 M NaOH was added until the desired pH (6.0) was attained. The material was filtered. The filtrate was electrodialysed in two steps. The final desalinate was applied to a column of Dowex HCR-W2 (1.5 cm × 30 cm, H^+ form, 20–50 mesh), followed by a column of Dowex 1-X8 (1.5 cm × 30 cm, formate form, 200–400 mesh). The latter column was washed with water, and thus eluted with a linear gradient of HCO_2H of 0–2 N at a flow rate of 2 ml min^{-1} [10, 16]. The Neu5Ac fractions were collected and evaporated at 45 °C under reduced pressure. The residue was decolorized with activated charcoal and then lyophilized.

2. Large scale. The milling of DEY powder was performed with a power mill (Type P-3, screen size of ϕ 16 mm; Showa Giken Co. Ltd, Osaka, Japan). The fine powdered DEY (500 kg containing 0.2% Neu5Ac by weight) was hydrolysed with HCl (pH 1.4) at 80 °C and neutralized with NaOH (pH 6.0). The mixture was filtered. The filtered DEY adjusted to the desired pH was electrodialysed in two steps using TS-10-360 (Tokuyama Soda Co. Ltd, Tokyo, Japan) for large scale preparation of Neu5Ac. The electrodialysed solution was applied to a column of Dowex HCR-W2 (25 cm × 40 cm, H^+ form, 20–50 mesh), followed by a column of Dowex 1-X8 (35 cm × 50 cm, formate form 200–400 mesh). The latter column was washed with water, and then eluted with a linear gradient of HCO_2H (0–2M). The eluates containing Neu5Ac were concentrated using a reverse osmosis membrane (NTR-7250; Nitto Denko Co. Ltd, Tokyo, Japan). The concentrate was finally rotary evaporated at 40 °C. The residue was then lyophilized to yield 500 g Neu5Ac, giving a 50% yield (Fig. 1). The purity of the Neu5Ac was >98% (TBA method). The purity of the compound was confirmed by TLC, NMR, IR and HPLC.

Results

Composition of DEY

The DEY, a byproduct from egg processing plants, was analysed. The DEY contained 55.4% water, 36.4% proteins 6.6% lipids (polar lipids 6.3%, nonpolar lipids 0.3%) and 1.6% total sugars including 0.2% Neu5Ac.

Effect of pH on the hydrolysed DEY

The effect of pH on the hydrolysed DEY on the Neu5Ac purity and adsorption capacity to Dowex 1-X8 anion column was examined. The eluates obtained from the

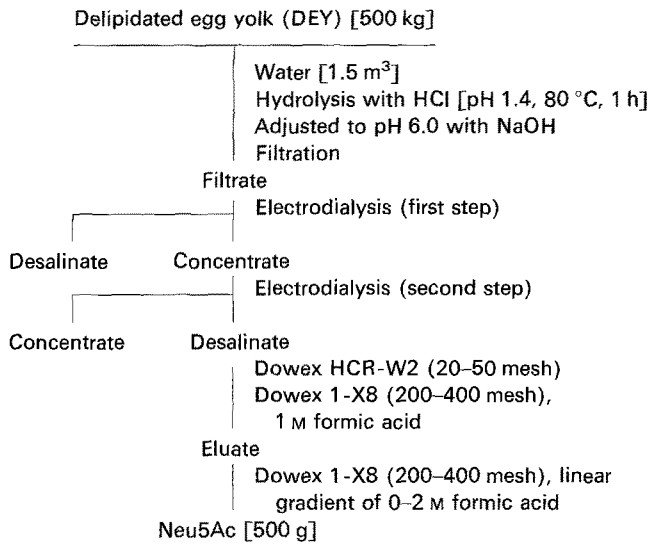

Figure 1. Large-scale preparation of Neu5Ac from the DEY.

hydrolysed DEY samples adjusted to pH 6.0 showed the highest purity (55%) and adsorption (27%).

Electrodialysis of the hydrolysed DEY prior to column chromatography

The hydrolysed DEY adjusted to pH 6.0 was electrodialysed to remove salts and other compounds to improve resin adsorption capacity of Neu5Ac. Electrodialysis of the DEY was performed in two steps with different ion exchange membranes. First step electrodialysis, using NEOSEPTA CM-1 and AFN membranes, was carried out for 60 min until the conductivity was 70 μS cm^{-1}. The charged compounds having a molecular weight of up to approximately 350, e.g., Neu5Ac, salts, amino acids etc., were present in the concentrate portion, and the desalinate contained high molecular weight compounds. The concentrate obtained in the first step was subjected again to electrodialysis using NEOSEPTA CM-2 and ACS membranes until the conductivity was 240 μS cm^{-1}. Almost complete recovery of Neu5Ac in the desalinate was obtained (Fig. 2).

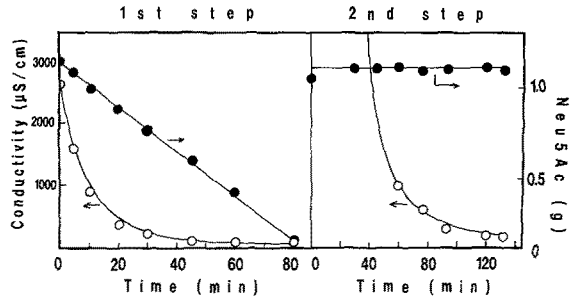

Figure 2. Time-course of two step electrodialysis of the hydrolysed DEY: ○, conductivity (μS cm^{-1}; ●, Neu5Ac (g).

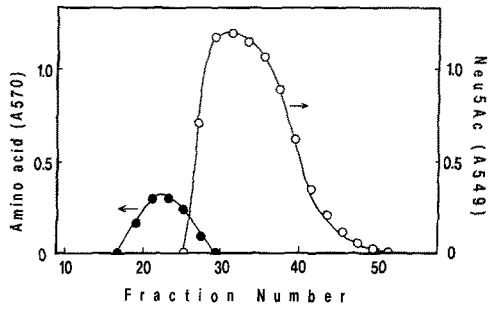

Figure 3. Elution profile of Neu5Ac from a Dowex 1-X8 anion exchange column. Procedure as given in Fig. 1. Neu5Ac content was measured by the TBA method. Amino acids were measured by the ninhydrin method. ●, Amino acid; ○, Neu5Ac.

Anion exchange chromatography of the electrodialysed DEY

The desalinated DEY obtained after the second step of electrodialysis was applied on anion exchange resin Dowex 1-X8 (1.5 cm × 30 cm, formate form) column. The column was washed with water and elution of Neu5Ac was carried out with 1 M HCO_2H. The eluates obtained were analysed for Neu5Ac (TBA method), and amino acids. The elution profile of Neu5Ac from the Dowex 1-X8 column showed that the contaminants, including amino acids, were eluted before Neu5Ac, and a significant proportion of the contaminants overlapped the Neu5Ac peak (Fig. 3).

The major amino acid contaminants in the hydrolysed DEY were glutamic acid and aspartic acid (Table 1).

The amino acid composition of the concentrated eluates after the first anion chromatography step showed that glutamic acid was by far the major remaining contaminant (Table 1).

Cation exchange chromatography prior to anion exchange column chromatography

Cation exchange chromatography was performed to remove compounds like glutamic acid which interfered with anion exchange chromatography. The hydrolysed DEY adjusted to pH 6.0 was electrodialysed in two steps. The filtrate was applied to a column of Dowex HCR-W2 followed by Dowex 1-X8 and rechromatographed on a Dowex 1-X8 column, yielding 99% pure Neu5Ac. The purity of Neu5Ac was decreased to 94% when no cation exchange column was used (Table 2).

Purity of sialic acid

1. TLC method. TLC of sialic acid liberated from the DEY fractions showed the Neu5Ac was the sole type of Neu5Ac present in the egg; no *N*-glycolyl derivative was found in the compound (Fig. 4).

2. NMR. The purity of Neu5Ac was confirmed from ^1H- and ^{13}C-NMR spectra. The ^1H- and ^{13}C-NMR spectra were measured in ^2H_2O at 400 MHz and 100 MHz,

Table 1. Amino acid composition of the hydrolysed DEY and the first chromatography eluates.

Amino acid	*Hydrolysed DEY*[a] (mol%)	*Eluates of first chromatography*[b] (mol%)
Asp	11.45	9.76
Thr	6.76	2.65
Ser	7.34	3.62
Glu	13.40	55.85
Pro	4.10	2.03
Gly	5.07	3.21
Ala	7.33	2.69
Cys	1.79	0.62
Val	7.26	1.86
Met	2.32	0.41
Ile	4.56	2.28
Leu	7.80	1.07
Tyr	3.52	0.62
Phe	3.10	2.79
Lys	6.21	5.34
His	2.40	4.34
Arg	4.10	2.03

[a] After electrodialysis.
[b] The concentration eluates obtained after first chromatography of Dowex 1-X8 (200–400 mesh).

Table 2. Purification of Neu5Ac by ion exchange chromatography.

Column resin *First chromatography*	*Dowex HCR-W2* *Dowex 1-X8*	*Dowex 1-X8*
Purity of Neu5Ac (%) [First chromatography]	84	75
Purity of Neu5Ac (%) [Rechromatography][a]	99	94

[a] Rechromatography was carried out using Dowex 1-X8.

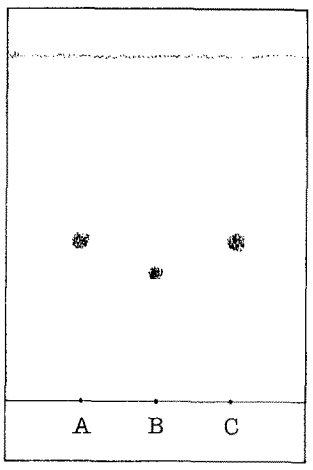

Figure 4. TLC chromatogram of sialic acids: A, authentic Neu5Ac; B, authentic Neu5Gc; C, sialic acid from large scale process.

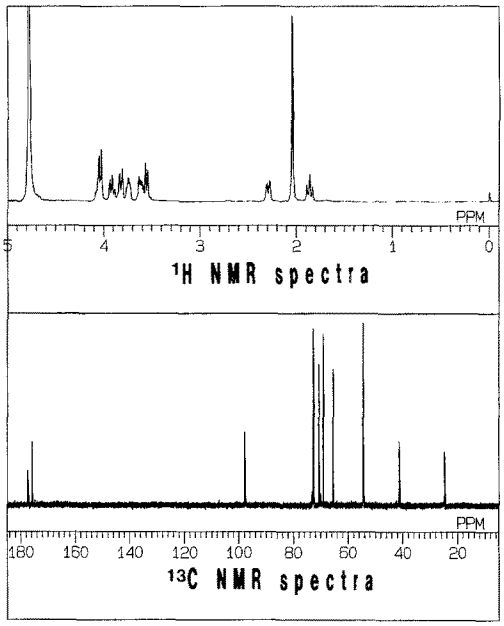

Figure 5. ^{1}H- and ^{13}C-NMR spectra of sialic acid. Solvent, ^2H_2O; internal standard, sodium 4,4-dimethyl-4-silapentanesulfonate.

respectively (JEOL GSX-400 instrument; JEOL Co. Ltd, Tokyo, Japan). The spectra matched those of authentic Neu5Ac (Fig. 5).

Discussion

Egg yolk is commonly processed for lipid extraction on an industrial scale. Delipidated yolk is discarded without further utilization. Quantitative estimation of various egg fractions showed that egg yolk contains the highest amount of Neu5Ac on a total egg weight basis [10] but usually its processing is tedious due to the large amount of lipids and various ionic compounds present in the fraction. The presence of lipids leads to emulsification of yolk during processing prior to the isolation of Neu5Ac. The DEY material used in this study was attractive for Neu5Ac preparation because of the very small amount of lipids present.

In our previous report on Neu5Ac preparation, the chalaza and egg yolk membrane mix was hydrolysed with H_2SO_4 and neutralized with saturated $Ba(OH)_2$ solution. Although the Barium is almost totally removed, even small traces left in the final product could be toxic for food or pharmaceutical purposes. In the present investigation, Neu5Ac was released from glycosidic linkages by HCl (pH 1.4) at 80 °C and the pH of the hydrolysed DEY was adjusted with NaOH, and the results were as good as when H_2SO_4 hydrolysis was used. The effect of pH on the hydrolysed DEY was examined for its anion-exchange adsorption efficiency and the purity of eluates. The maximum adsorption and the highest purity was obtained

at pH 6.0. Some of the high molecular weight compounds, salts and remaining lipids were separated from Neu5Ac by electrodialysis.

Although two step electrodialysis using cation and anion exchange membranes was very effective in removing the large amount of contaminants interfering in anion chromatographic purification of Neu5Ac, the final desalinate solution still contained charged low molecular weight compounds. The major contaminant was found to be glutamic acid. The cation exchange Dowex HCR-W2 (20–50 mesh) column used prior to anion exchange Dowex 1-X8 (200–400 mesh) column removed major contaminants like glutamic acid. Using this procedure, we achieved the purification of Neu5Ac to >98% and 500 g Neu5Ac was obtained from 500 kg DEY (Fig. 1).

TLC and NMR spectra of the compound confirmed that there was no glycolyl or *O*-acetyl group in the compound. Concentration using a reverse osmosis membrane is carried out at room temperature and is a very fast and efficient technique compared to rotary evaporation to concentrate large amounts of Neu5Ac eluates obtained during column chromatography.

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