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Note

Large-scale preparation of sialic acid from chalaza and egg-yolk membrane*

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Many derivatives of sialic acids are present in mammalian tissues, in vertebrates, and in bacteria as components of glycoproteins, glycolipids, oligosaccharides, and also in the free state[1]. Although the family of sialic acids has grown to more than 30 members, the most widely distributed sialic acid is *N*-acetylneuraminic acid (Neu5Ac) followed by *N*-glycolylneuraminic acid (Neu5Gc)[2].

Sialic acid and its derivatives have been shown to be involved in the regulation of many physiological processes[3]. In the past two decades, many reports have been published on the biological role of sialic acids[4–11].

In order to fulfil increasing demands for sialic acid, its economical large-scale preparation is desired. Only a few natural sources, such as swallow's nest, milk protein[12], and a microbial fermentation process[13] have been explored for practical, industrial-scale preparation of sialic acid.

The avian egg is considered to be a chemical storehouse and is composed of substances that form the basis of life[14]. Along the egg's long axis, a ropy structure of cloudy appearance spirals from the yolk into albumen at each end of the egg (Fig. 1a). These two structures are called chalazae (Fig. 1b). Chalaza has been reported to be rich in sialic acid[15].

We compared the Neu5Ac contents of chalaza and egg-yolk membrane in various species of avian eggs, namely hen, quail, pigeon, and gamecock (Table I). The highest Neu5Ac content in the egg-yolk membrane (2.19%) and chalaza (3.21%) were found in gamecock and pigeon, respectively.

Measurement of the Neu5Ac content in various fractions of hen's egg (fresh egg) showed that Neu5Ac is distributed in all parts of the egg, but is mainly located in chalaza and egg-yolk membrane (Table II). Egg white and ovomucin (isolated from egg white)

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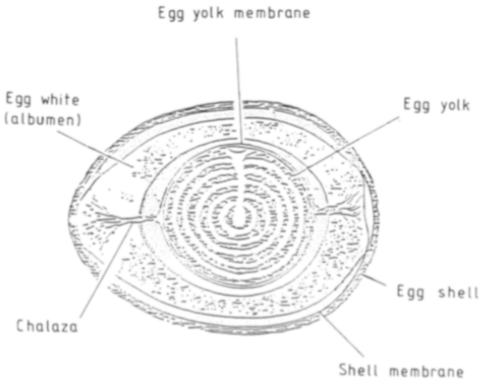

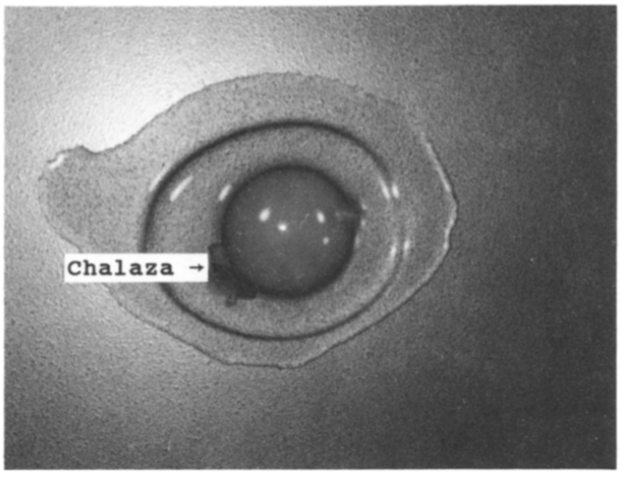

Fig. 1. (a) Structure of the hen's egg, shown by a section through the long axis; (b) broken egg, showing chalaza.

TABLE I

Sialic acid content in various species of avian egg

Avian egg species	*Egg-yolk membrane (% in dry matter)*	*Chalaza (% in dry matter)*
Hen (*Gallus domesticus*)	1.0	2.40
Quail (*Coturnix coturnix japonica*)	1.25	3.79
Pigeon (*Columba livia*)	1.85	3.21
Gamecock (Japanese Bantam) (*Gallus domestica*)	2.19	2.68

were found to contain 0.1 and 2% of Neu5Ac, respectively, but only a small quantity of ovomucin (0.9%) was obtained from egg white. Moreover, it is difficult to isolate ovomucin from egg white. The egg-yolk fraction contains a significant proportion of

TABLE II

Sialic acid contents in various fractions of hen's egg

Fractions	*Qty.*	*Sialic acid*	
	(Kg/ton egg wet wt. basis)	*(g)*	*(% in dry matter)*
Egg shell	104.8	2.98	0.004
Shell membrane	6.2	1.22	0.07
Egg white	603.5	60.35	0.10
Egg yolk	281.0	267.0	0.19
Egg-yolk membrane	2.3	3.52	1.80
Chalaza	2.2	3.96	2.40

TABLE III

Sialic acid contents in different organs of hen

Organ	*Sialic acid (% wet wt. basis)*
Oviduct	0.017
Ovary	0.076
Crest	0

Neu5Ac (0.19%). Although egg yolk and egg white may be utilized as raw materials for preparation of sialic acid, the processing of these fractions is tedious and uneconomical, because large quantities of raw materials have to be used as compared to chalaza and egg-yolk membrane. Furthermore, egg yolk has to be delipidated to avoid emulsification prior to isolation of sialic acid.

To screen alternative Neu5Ac-rich material, some of the reproductive organs and the crest of the hen were examined. The Neu5Ac content of ovary was also high (0.076% on wet wt. basis, Table III), but it is difficult to collect large amount of reproductive organs as byproducts for industrial-scale production of sialic acid. No Neu5Ac was detected in crest.

In egg-processing factories, chalaza along with egg-yolk membrane fractions are separated off from eggs by filtration prior to the processing of egg products, and are usually discarded without further utilization. In the present investigation, hen's egg chalaza and egg-yolk membrane mixture (a byproduct of the egg-processing plant of Taiyo Kagaku Co.) were used as a raw material for sialic acid preparation (Fig. 2).

The Neu5Ac content (430 g in a 125-kg batch of washed raw material containing chalaza and egg-yolk membrane) was 0.34% (wet wt. basis), as compared to the content of 0.15% in egg-yolk membrane (wet wt. basis) and 0.18% in chalaza (wet wt. basis) from fresh egg on the laboratory scale. The increased Neu5Ac content might be attributable to the processing of raw material through a screw decanter. Approximately

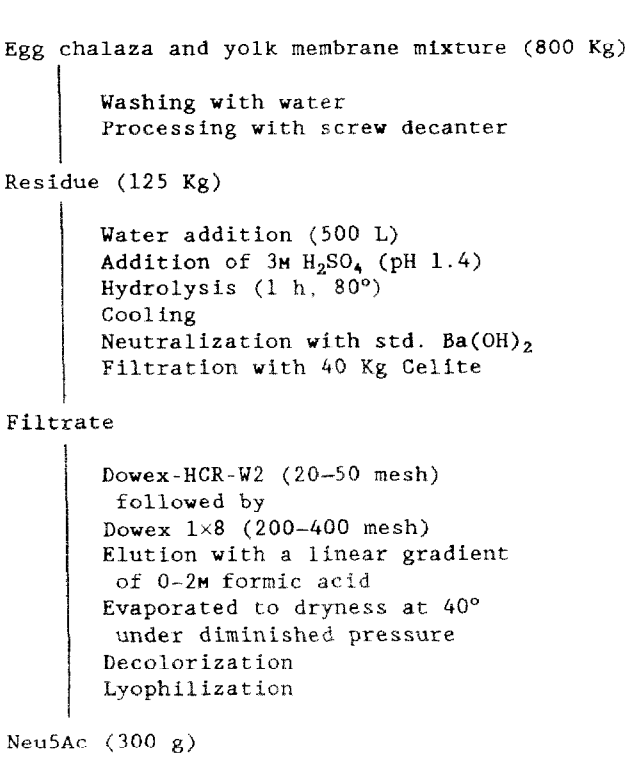

Fig. 2. Process flow of large-scale preparation of sialic acid.

300 g of Neu5Ac was obtained in one batch and its purity was >97% (TBA method). The yield of Neu5Ac obtained after final purification was obtained after final purification was 70%, based on the weight of washed egg chalaza and membrane mixture.

T.l.c. of sialic acid liberated from the egg fractions showed that Neu5Ac was the sole type of sialic acid present in the egg; no spots other than Neu5Ac were detected (Fig. 3). The purity of Neu5Ac was confirmed by h.p.l.c. (Fig. 4), i.r. spectra (Fig. 5), and ^{13}C-n.m.r. spectra (Fig. 6). The spectra matched well with those of authentic Neu5Ac.

The chemical synthesis of sialic acids is complicated and expensive, and thus its isolation from natural sources is desired. Although the Neu5Ac content among the avian species examined was the highest in quail's egg chalaza, hen eggs were used for large-scale preparation because of their availablity and utilization all over the world. Chalaza and egg-yolk membrane are usually discarded in egg-processing plants and are thus easily obtained. These fractions can therefore be used as an excellent source for cheap industrial-scale preparation of large amounts of Neu5Ac. Preparation of Neu5Ac from these materials is also very attractive because of the convenient purification procedure.

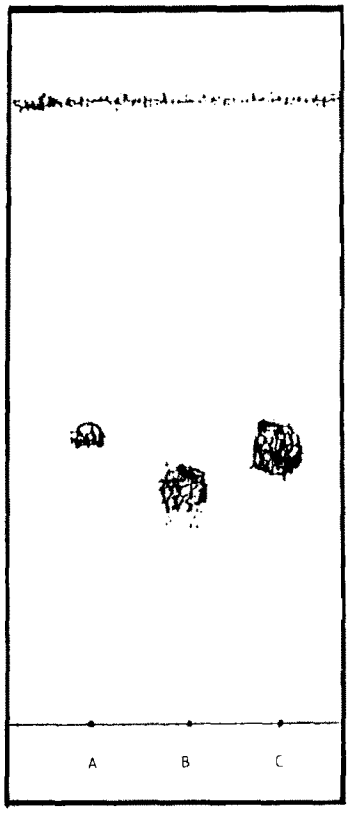

Fig. 3. T.l.c. chromatogram of sialic acids. [A: Authentic Neu5Ac; B: authentic Neu5Gc; C: sialic acid from large-scale process.]

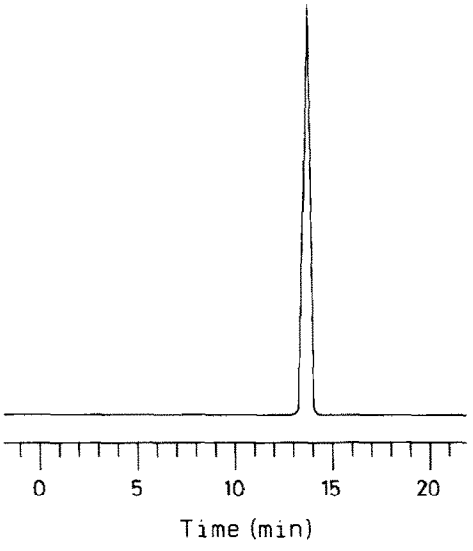

Fig. 4. H.p.l.c. chromatogram of sialic acid obtained from large-scale process.

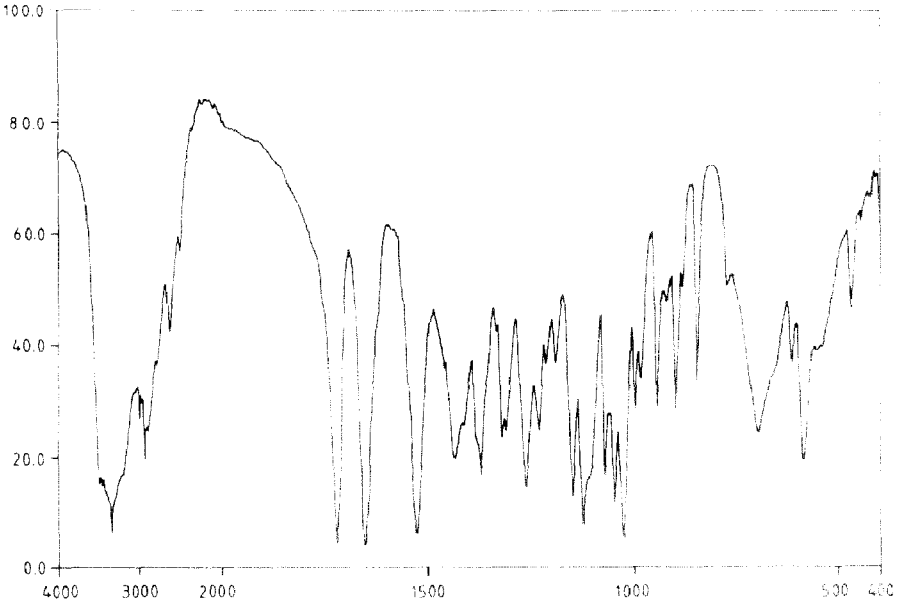

Fig. 5. ir. spectrum of sialic acid obtained from large-scale process.

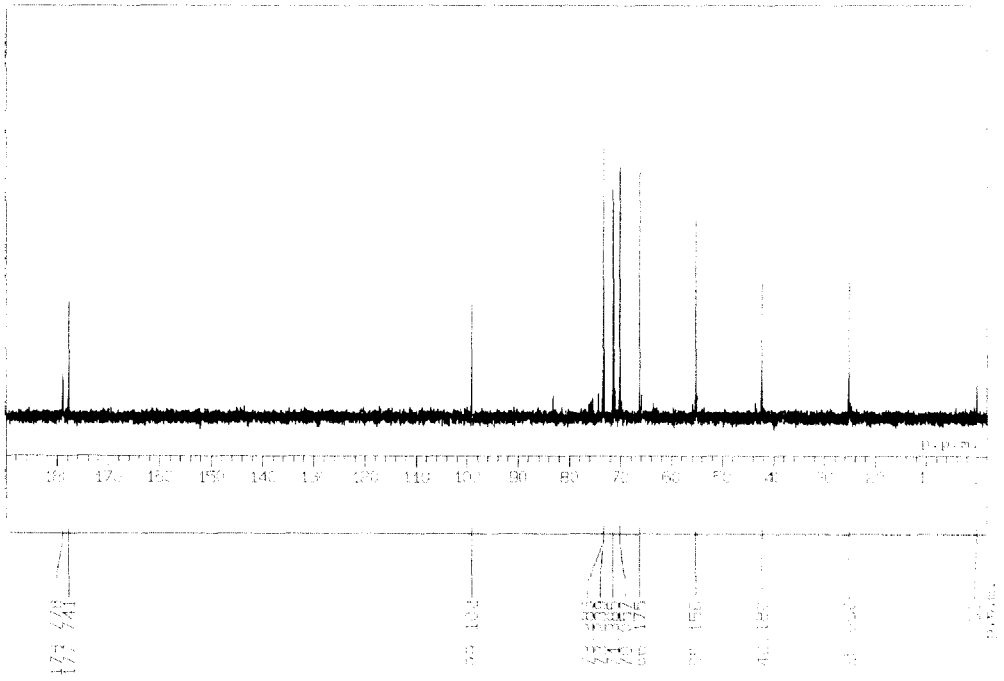

Fig. 6. ^{13}C-N-m.r. spectrum of sialic acid obtained from large-scale process [solvent, D_2O; internal standard, sodium 4,4-dimethyl-4-silapentanesulfonate]

EXPERIMENTAL

Analysis of sialic acid. — Sialic acid was quantitated by the modified periodate thiobarbituric acid (TBA)[16] and periodate–resorcinol methods[17]. Sialic acid liberated by heating the materials in 0.05M H_2SO_4 at 80° was monitored colorimetrically. Authentic Neu5Ac from *E. coli* was obtained from Nakarai Tesque Inc. (Kyoto, Japan)[13]. Authentic Neu5Gc (from porcine submaxillary glands) was purchased from Sigma Chemical Co. (St. Louis, MO, U.S.A.).

T.l.c. employed (6:2:1) 1-propanol–M ammonium hydroxide–water as developer with aluminium sheets of silica gel 60 (Merck)[18]; detection was achieved by spraying with 5% H_2SO_4 in MeOH and heating for 5 min at 150°.

H.p.l.c. was performed with an Eyela PLC-5D (Tokyo Rikakikai Co., Ltd., Tokyo, Japan) instrument 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 (5mM H_2SO_4) flowed at 0.65 mL/min, under a pressure of 57 kg/cm^2 at ambient temperature.

I.r. spectra (KBr) of sialic acid samples were recorded with a Shimadzu IR 460 spectrometer (Shimadzu Corporation, Kyoto, Japan). ^{13}C-N.m.r. spectra was measured in D_2O at 100 MHz by a Jeol GSX-400 instrument (Jeol Ltd., Tokyo, Japan) operated in the pulsed Fourier-transform mode. Sodium 4,4-dimethyl-4-silapentanesulfonate was used as the internal standard.

Laboratory scale. — Fresh eggs were separated into egg white and egg yolk. Various fractions of egg, namely shell, shell membrane, egg white, egg yolk, yolk membrane, and chalaza were collected. Chalaza was carefully picked out with tweezers and incubated with 3 volumes of 2% KCl for 2 days at 5° and later washed with water to remove contamination by egg white and egg yolk. The chalaza fraction thus purified was collected. The chalaza cords separated from the egg yolks were squeezed through a cloth for collection of the egg-yolk membrane fraction. The egg-yolk membranes remaining on the cloth were purified by repeatedly washing with water. The fractions of egg white, egg yolk, yolk membrane, chalaza, shell membrane, and egg shell collected were finally vacuum dried and pulverized. Egg-yolk membrane and chalaza of hen, quail, pigeon, and gamecock were collected as already mentioned and their Neu5Ac contents determined.

Hens were sacrificed and various organs, namely ovary, oviduct, and crest were collected. The organs were minced and hydrolyzed, and the Neu5Ac contents of the hydrolyzed organs were measured.

Large-scale. — Hen eggs were separated into egg yolk and egg white with an egg processor and passed through a stainless-steel filter (mesh size = 0.8–1.0 mm). The chalaza mixed with yolk membrane entrapped in the filter were scraped, collected, and used for large-scale preparation of Neu5Ac (Fig. 2).

The crude mixture of egg chalaza and yolk membrane (800 kg) was washed with water to roughly remove contamination by egg white and egg yolk, and the residue (125 kg) was homogenized with three volumes of water. The suspension was acidified to

pH 1.4 with 3M H_2SO_4 and heated for 1 h at 80°. After cooling, saturated $Ba(OH)_2$ solution was added until pH 5.0 was attained, and the mixture was filtered. The filtrate was applied to a column of Dowex HCR-W2 (20–50 mesh), followed by a column of Dowex 1 × 8 (200–400 mesh). The latter column was washed with water, and then eluted with a linear gradient of HCO_2H from 0 to 2M. The Neu5Ac was eluted over a narrow range at ~0.8M concentration of HCO_2H. The eluates containing Neu5Ac were evaporated to dryness at 40° under diminished pressure. The residue obtained was decolorized with activated charcoal powder and then lyophilized.

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