SEPARATION OF SENDAI VIRUS GLYCOPROTEINS BY CM–SEPHAROSE COLUMN CHROMATOGRAPHY

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1. Introduction

Sendai virus contains two glycoproteins, hemagglutinin-neuraminidase (HN) and fusion (F) protein [1]. We were interested in separating these glycoproteins in sufficiently pure form to specify immune responses. During fetuin affinity chromatography [2] and electrofocusing [3], we obtained some neuraminidase activity in the F-containing fraction. Moreover zonal sedimentation [4,5] and lectin affinity chromatography [6,7] do not seem to be satisfactory for separation of paramyxovirus glycoproteins. We have developed a method for separation of Sendai virus glycoproteins with glutaraldehyde-treated red blood cells. This method is convenient for separation of HN protein, but yields an F fraction which is sometimes contaminated with red cell materials.

A pure HN fraction by chromatography was obtained on a DEAE Bio-Gel A column of an anion exchanger type [8,9]. So we tried to obtain a pure F fraction by chromatography on a CM–Sepharose column of a cation exchanger type. By combining the two chromatographies we obtained pure F and HN fractions. Here we report our method and some properties of the glycoproteins separated.

2. Materials and methods

Sendai virus, Z strain, was grown in 10-day-old embryonated eggs and purified by velocity sedimentation in sucrose gradients [10]. The visible bands of virus were separated, and the suspension medium was replaced by 5 mM Tris–HCl (pH 7.2) after precipitating the virus by ultracentrifugation. The purified virions were disrupted by treatment with 2% (v/v) Triton X-100 in 5 mM Tris–HCl (pH 7.2) at 25°C for 15 min, and centrifuged at 100 000 × *g* for 1 h. The resulting supernatant was dialyzed against 10 mM sodium acetate (pH 6.0)–0.25% Triton X-100 (SAT buffer) for 2 h, then loaded onto a column of CM–Sepharose CL-6B. Elution was done with a linear gradient of NaCl or with 0.25 M NaCl in SAT buffer. The fractionated proteins were concentrated by dialysis against saturated poly(ethylene glycol) (20 000) in 50 mM Tris–HCl (pH 7.2). Triton X-100 was partially removed from the protein fractions by dialysis against phosphate-buffered saline (PBS) containing Bio-Beads SM2 [11] for several hours, and then by Bio-Gel A 1.5m gel filtration. DEAE Bio-Gel A column chromatography was done as in [8].

Membrane particles (liposomes) were reconstituted by the dialysis method [12]. Glycoproteins and lipids (Sendai virus total lipids, generously given by Dr Keizo Inoue, Faculty of Pharmacy, Tokyo University) were each dissolved in 0.25% Nonidet P40 and mixed at a protein to lipid ratio of 0.5 (w/w). The mixture was put into Spectropore membrane tubing 2 (Spectrum Medical Ind.) and dialyzed against PBS in the cold for 3 days, with frequent changes of the PBS. Hemolysis assay was carried out using ^{51}Cr-labeled chicken red blood cells [13]; 50 μl aliquots of test samples were mixed with the cells (1.6×10^4 cpm/1.8×10^6 cells) and stood for 40 min at 0°C. Then the mixtures (total volume adjusted to 600 μl with PBS) were incubated at 37°C for 2.5 h. The cells were precipitated by centrifugation and 300 μl of the supernatants were taken for determination of released ^{51}Cr in a gamma counter. Percentage hemolysis was calculated as follows:

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% Hemolysis = [^{51}Cr release with the sample – ^{51}Cr release with PBS]

× 100/[Total ^{51}Cr count – ^{51}Cr release with PBS]

Sodium dodecyl sulfate (SDS)–polyacrylamide gel electrophoresis was run in 8% slab gels by the Laemmli method [14]. Protein was measured by the Lowry method [15]. Neuraminidase activity, hemagglutination and hemagglutination inhibition (HI) were measured as in [10,16]. Phosphate content was determined as in [17]. Rabbit anti-F and anti-HN antisera were prepared by intramuscular and intravenous injections, respectively, of the separated F and HN proteins 3 times without adjuvant. The gel diffusion test was done in 1% agarose A-37 (Nakarai Chem.) containing 0.01% sodium azide.

3. Results

The gel electrophoresis patterns of the Triton-solubilized supernatant (fig.1) showed that the supernatant consisted essentially of two glycoproteins, HN and F, although a faint band was observed in the position of nucleoprotein (NP). On CM–Sepharose column chromatography (fig. 2), the first protein peak without neuraminidase activity was observed in the unadsorbed fraction, and the second peak with high neuraminidase activity was eluted with 0.15–0.20 M NaCl. SDS gel electrophoresis (not

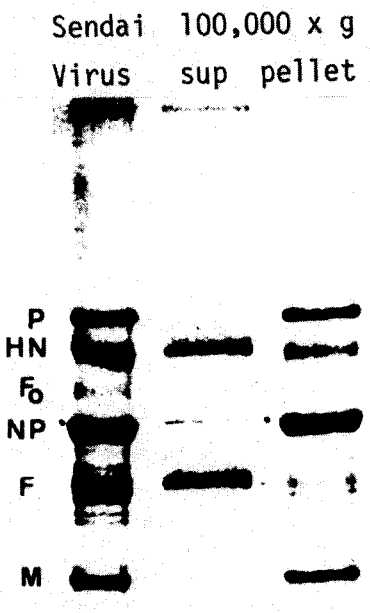

Fig.1. SDS gel electrophoresis patterns of Sendai virus, Triton-solubilized supernatant and pellet fractions, separated by centrifugation at 100 000 × *g* for 1 h. Coomassie brilliant blue staining. Bands are designated as in [1].

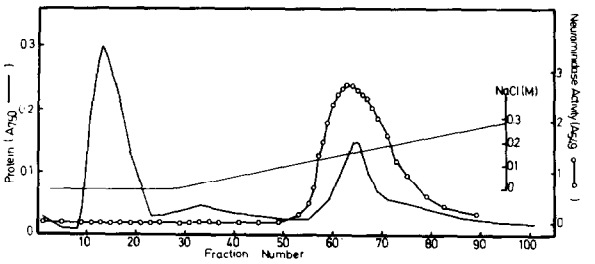

Fig.2. Separation of neuraminidase negative (F) and positive (HN) protein fractions by CM–Sepharose column chromatography. The Triton-solubilized supernatant (30 ml) containing 15 mg protein was applied to a column (2.6 × 30 cm). Fractions of 6 ml were collected. Aliquots of 20 μl and 200 μl were assayed for neuraminidase activity and protein, respectively.

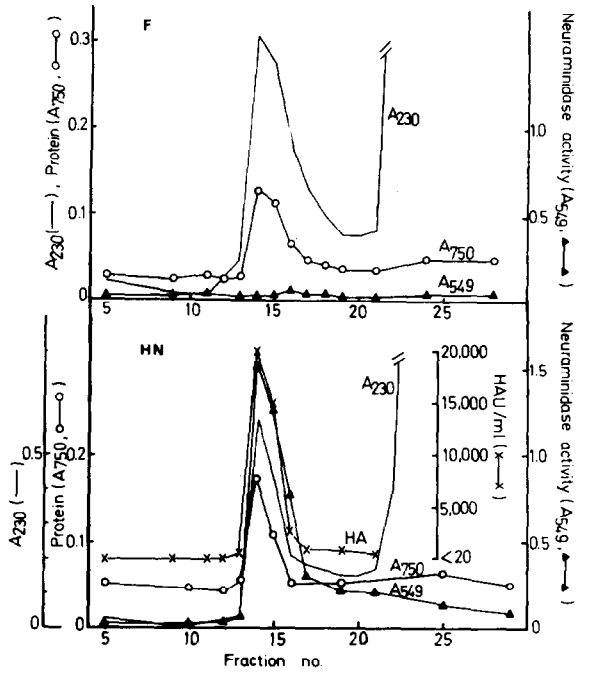

Fig.3. Gel filtration patterns of F and HN proteins obtained from CM–Sepharose, on a Bio-Gel A 1.5m column (1.6 × 96 cm). Elution was done with 10 mM potassium phosphate (pH 7.2). Fraction volumes and assay methods were as in fig.2. The A_{230} was measured for both protein and Triton X-100.

shown) revealed that the first peak contained F protein and the second peak HN protein. The two peak fractions were each concentrated, and dialyzed to remove the detergent partially. Then they were applied one after the other to a column of Bio-Gel A 1.5m (fig.3). In both cases, the protein was recovered in the void volume with ~90% recovery. Most of the detergent was removed from the protein fractions by gel filtration. The patterns of these fractions on gel electrophoresis (fig.4) showed that they contained only F and HN protein, respectively. Phosphate measurement of the F and HN fractions obtained by gel filtration showed that they contained ~13 and 9 mol phosphate/mol protein, respectively, suggesting that they still contained some phospholipid. Most of the viral lipids, which had been recovered in the unadsorbed fraction of CM–Sepharose chromatography together with F protein, was removed from the F fraction.

Sometimes, the leading shoulder of the second peak (HN protein) shown in fig.2 was found to be contaminated with a trace of F protein. In such cases, the HN fraction was purified further by DEAE Bio-Gel column chromatography (see table 1 and section 4) and then gel filtration. Fig.5A,B are electron micrographs of the F fraction purified by CM–Sepharose chromatography and the HN fraction purified by CM–Sepharose and DEAE Bio-Gel. The former consisted of aggregates of straight spikes of 14 nm length (the length does not include the base structure), with a knob at the top, and some vesicular structures, which were possibly contaminating lipids. The latter consisted of aggregates of fibrous spikes of ~11–12 nm length, some of which were not clearly resolved. The HN spikes also had a knob at the top. These morphological features of F and HN spikes were consistent with findings on SV spikes [18] and Sendai virus spikes [3].

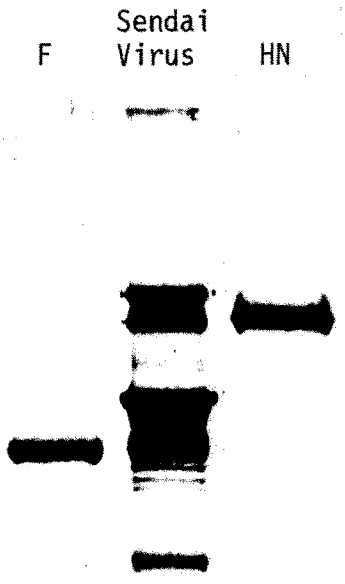

Fig.4. SDS gel electrophoresis patterns of purified F and HN proteins, applied at 6 μg and 9 μg, respectively.

Table 1
Hemolytic activities of reconstituted liposomes

Samples	Protein (μg)	% Hemolysis	
		– WGL	+ WGL[a]
1. F liposomes	2.5	1.3	19.8
2. HN liposomes	2.5	6.0	0.0
3. F + HN liposomes	2.5	82.0	87.4
4. F + HN, lipid⁻	2.5	14.7	6.4
5. Lipid alone	0.0	–0.76	0.41
6. Sendai virus	5 HAU[b]	25.9	13.1
	10 HAU	40.9	19.1

[a] Wheat germ lectin (4.5 μg/ml)
[b] Hemagglutination units of infected chorioallantoic fluid

Purified F, HN or F 50 μg plus HN (25 μg + 25 μg) was mixed with 100 μg Sendai virus total lipids in 0.25% Nonidet P40 and liposomes were reconstituted as in section 2. Proteins (F + HN) without lipid and lipid without protein were treated similarly as controls. For this experiment, HN protein eluted stepwise from CM–Sepharose with 0.25 M NaCl in SAT buffer was purified further by passing it through a DEAE Bio-Gel column [8] equilibrated with 10 mM sodium phosphate (pH 7.2)–0.1% Triton X-100

The HN protein obtained was biologically active, since it had neuraminidase and hemagglutinating activities (fig.3). To see whether isolated F protein was biologically active, membrane particles (liposomes) were reconstituted from F protein with or without HN protein, and their hemolytic activities were examined. F liposomes had no hemolytic activity, but F + HN liposomes had high activity (table 1, left column). HN liposomes had no hemolytic activity. Since, F liposomes were found to have no hemagglutinating activity, no attachment function, an external attachment factor, wheat germ lectin (WGL), was added to the assay system [19,20]. In the presence of WGL, the F liposomes manifested hemolytic activity (table 1, right column), although much less than that of F + HN liposomes.

WGL inhibited the hemolytic activity of intact Sendai virus. The finding that WGL did not inhibit the hemolytic activity of F + HN liposomes may indicate that it manifested the hemolytic activity of non-functional F liposome-like particles included in the

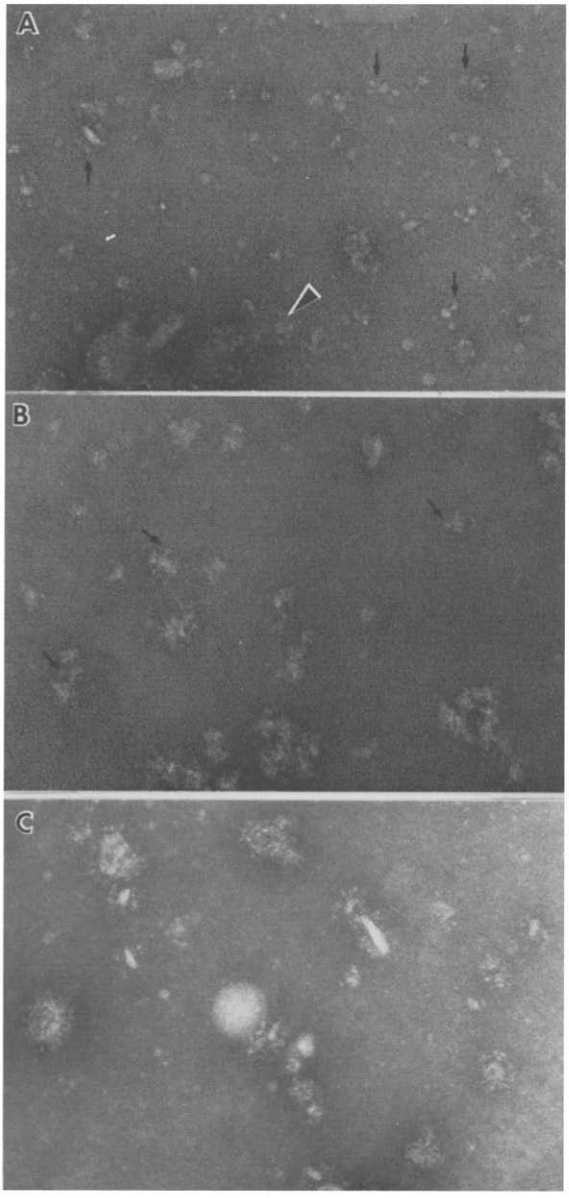

Fig.5. Electron micrographs of F (A), HN (B) fractions, and F + HN liposomes (C). Negative staining with 1% uranyl acetate (A,B) or 2% phosphotungstic acid (C). Arrows and the arrow head indicate spikes and vesicular structures, respectively. × 114 000.

Fig.6. Gel diffusion patterns of anti-F and anti-HN rabbit sera against F and HN proteins.

F + HN liposome preparation and inhibited that of fully functional F + HN liposomes, and that these effects counteracted each other. Reconstitution of F and HN proteins without additional lipid resulted in formation of liposomes with low but significant activity, possibly due to the contribution of the small amounts of lipids contaminating the F and HN preparations. All these findings indicate that the F protein obtained was biologically active. Fig.5C is an electron micrograph of F + HN liposomes, which look like fragments of virus envelopes with spikes and are similar to reconstituted Sendai virus envelope particles [12].

Rabbit anti-F and anti-HN sera exhibited monospecificity against F and HN preparations respectively in gel diffusion tests (fig.6). The HI titer of anti-HN serum was 5000. Some anti-F sera had low HI titers (<10–40), but they did not inhibit the neuraminidase activity of purified HN protein or Sendai virus (not shown), suggesting that the low HI titers were not due to the contamination of anti-HN antibodies.

4. Discussion

Here we prepared a highly pure F glycoprotein fraction by CM–Sepharose column chromatography, and could reproducibly obtain monospecific anti-F rabbit sera using the purified F protein as immunogen. The HN protein fraction obtained by this method was sometimes contaminated with a trace of F protein. Hyperimmune sera, obtained by repeated injection of the HN fraction purified by CM–Sepharose chromatography into rabbits, usually contained antibodies

against F as well as against HN protein. However, on removal of the F contaminants by DEAE Bio-Gel chromatography, we could obtain monospecific anti-HN serum. If loss of one glycoprotein is not important, then it is better to prepare F and HN proteins separately, using CM–Sepharose and DEAE Bio-Gel columns, respectively.

The HN and F proteins thus prepared were biologically active. We do not know whether the mechanism of the lectin-mediated hemolytic activity of F liposomes is the same as that of F + HN liposomes or Sendai virus. Therefore, the most reliable method to check F function is probably to examine the activity of liposomes reconstituted from F protein together with HN protein.

This method provides a convenient and reliable way to prepare pure HN and F proteins, which can specify immune responses.

Acknowledgements

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