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Purification of Infectious Bronchitis Coronavirus by Sephacryl S-1000 Gel Chromatography

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ABSTRACT

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A procedure was developed to purify infectious bronchitis virus (IBV) by gel chromatography (GC) with a Sephacryl S-1000 column. Virus samples concentrated by centrifugation were applied to a Sephacryl S-1000 column and eluted by 0.02 M phosphate buffer (pH 7.2) containing 0.15 M NaCl. Virus particles were recovered mainly in the first peak. Purity of the samples was evaluated by both sodium dodecyl sulfate–polyacrylamide gel electrophoresis and electron microscopy. Using electron microscopy, it was found that there were more spike-rich particles in the virus samples purified by GC than in those purified by sucrose density gradient centrifugation (SDGC). In addition, the hemagglutination unit [$\log_{10}$ (infectivity titer/hemagglutination titer)] of GC-purified virus samples was ∼ 10 times lower than that of SDGC-purified virus samples. These results indicate that Sephacryl S-1000 gel chromatography is useful for purification of IBV.

INTRODUCTION

Infectious bronchitis virus (IBV) belongs to the Coronaviridae. The IBV virion with a diameter of ∼ 120 nm is enveloped and contains one molecule of an infectious (plus-sense), single-stranded RNA (Robb and Bond, 1979; Shiddell et al., 1982). The virion is mainly composed of spike (S; peplomer) with a molecular weight of 128 kDa, membrane (M) with a molecular weight of 45 kDa and nucleocapsid (N) with a molecular weight of 25 kDa (Spaan et al., 1988). S and M proteins are both glycosylated and parts of them are exposed at the surface of the virion. S protein consists of two glycoproteins, S1 and S2 (Cavanagh, 1981, 1983; Stern et al., 1982). The S protein of IBV may play an important role in viral activities, such as hemagglutination (HA), infectivity for cultured cells, and antigenicity recognized by neutralizing antibody (Cavanagh and Davis, 1986; Cavanagh et al., 1986). Therefore, it is necessary to obtain spike-rich virus preparations for some studies of IBV.

For purification of IBV, sucrose density gradient centrifugation (SDGC) has usually been employed (Cavanagh, 1981; Yagyu and Ohta, 1985). Recently, it was reported that binding of S1 and S2 was very weak (Stern and Sefton, 1982). More recently, Yagyu and Ohta (1985) showed that the spike protein was easily dissociated from the virion during the purification by SDGC in some vaccine strains employed in Japan. Accordingly, we attempted to apply gel chromatography (GC) using a Sephacryl S-1000 column for the purification of IBV, instead of using SDGC.

MATERIALS AND METHODS

Propagation of virus

Strain M41 of IBV was used. Virus was inoculated into the allantoic cavity of 11-day-old embryonated specific-pathogen-free (SPF) chicken eggs. The inoculated eggs were incubated at 37°C for 36–48 h and then chilled at 4°C overnight. Allantoic fluid was harvested from the eggs and clarified by centrifugation.

Infectivity assay

Virus infectivity was determined as follows. Serial 10-fold dilutions of virus sample made in phosphate-buffered saline (PBS, pH 7.4) were inoculated into 9-day-old embryonated SPF eggs (five eggs per dilution) which were incubated for 7 days after inoculation. The 50% egg infectivity dose (EID_{50}) was calculated by the method of Reed and Muench (1948).

Gel chromatography

Clarified allantoic fluid was concentrated by centrifugation at $50\,000 \times g$ for 2 h. The concentration virus was applied to a Sephacryl S-1000 (Pharmacia Fine Chemicals, Uppsala, Sweden) column (1.0 by 90 cm) equilibrated with 0.02 M phosphate buffer (pH 7.2) containing 0.15 M NaCl. The column was eluted with the same buffer at a flow rate of 6 ml h^{-1} and every 3 ml was fractionated. Fractions containing virus particles were pooled and concentrated by centrifugation.

Sucrose density gradient centrifugation

Concentrated virus was layered onto 10–50% discontinuous sucrose density gradients and spun at 27 000 rpm for 4 h (RPS 27-3 rotor, Hitachi, Japan). A band at 45% sucrose containing virus particles was collected, diluted with 0.15

M NaCl and centrifuged at 24 000 rpm for 1 h with the same rotor. The resulting pellet was suspended with a small volume of PBS.

Determination of specific activity

Protein concentration was determined by the method of Lowry et al. (1951), with bovine serum albumin as the standard. The specific activity of each virus sample was determined using the following formula:

$$\log_{10}(\text{total infectivity titer } (EID_{50} \text{ ml}^{-1})/\text{total protein (mg)})$$

Their values provide one of the criteria for purity of the purified virus samples in comparison with those of the starting materials.

Electron microscopy

The virus samples were mounted on carbon-coated grids and then stained negatively with 1% uranyl acetate (pH 7.2) for 1 min. The electron microscope was a Model 100S (Nihon Denshi, Japan) and operated at 80 kV. More than 100 virus particles were counted and the spike retention rate was expressed as the percentage of the particles with > 10 spikes on each particle.

Hemagglutination test

Concentrated or purified virus samples were treated with phospholipase C (PLC) type 1 from *Clostridium welchii* (Sigma, St. Louis, MO, 1 unit ml^{-1}), at 37°C for 1 h under conditions similar to those of Alexander and Chettle (1977). The HA test was carried out in V-bottomed 96-well microtiter plates. The PLC-treated virus sample was diluted in 2-fold steps with 0.05 ml of 0.01 M Tris–HCl buffer (pH 6.5). To each well, 0.05 ml of 1% chicken erythrocytes were added and mixed well. The mixture was incubated at 4°C for 1 h. The HA units were calculated according to the following formula:

$$\text{HA unit} = \log_{10}(\text{infectivity titer } (EID_{50} \text{ ml}^{-1})/\text{HA titer})$$

Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS–PAGE)

SDS–PAGE was carried out as described by Cavanagh (1981). Briefly, virus samples were treated with 1% SDS and 1% 2-mercaptoethanol for 1 min in boiling water. Virus protein was separated on 10% polyacrylamide slab gels with 3% stacking gels, using Laemmli's discontinuous buffer system (Laemmli, 1970). Electrophoresis was performed for 6–7 h at a constant current of 1.2 mA cm^{-1}. The gel was stained with a silver stain kit (Bio-Rad, Richmond, CA, U.S.A.) as described in the instruction manual. The following molecular weight

marker proteins were used: phosphorylase "b", 94 kDa; albumin, 67 kDa; oval-bumin, 43 kDa; carbonic anhydrase, 30 kDa.

RESULTS

Gel chromatography

The virus preparations of M41 strain concentrated by centrifugation were applied to a Sephacryl S-1000 column, and the elution profile obtained is shown in Fig. 1. Protein concentration was determined by absorbancy at 280 nm. Two peaks of absorbancy were observed; one was small and the other was very large. Virus particles were seen in only the first peak, as determined by electron microscopy. Therefore, viruses in the first peak were concentrated by centrifugation and used as a purified virus sample.

Hemagglutinating activity

The HA units of the GC-purified virus samples were compared with those of the SDGC-purified virus samples. The SDGC-purified virus samples showed 7.2 and 7.5 HA units, compared with the GC-purified virus samples of 6.1 and 6.4 HA units (Table 1).
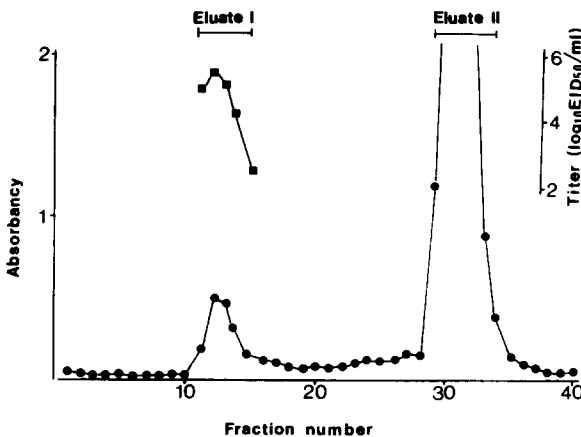

Fig. 1. Gel chromatography with a Sephacryl S-1000 column (1.0×90 cm) of IBV Strain M41. Concentrated virus was applied to the column and eluted at a flow rate of 6 ml h^{-1} with 0.02 M phosphate buffer (pH 7.2) containing 0.15 M NaCl. Absorbancy of each fraction was measured at a wavelength of 280 nm (●) and the infectivity titer determined (■).

TABLE 1

Comparison of two methods of purification with regard to hemagglutination (HA) activity

Experiment No.	Method	Infectivity titer[1] $(EID_{50} \, ml^{-1})$ (A)	HA titer[2] (B)	HA unit $(\log_{10} A/B)$
1	Sucrose density gradient centrifugation	$10^{8.7}$	32	7.2
	Gel chromatography	$10^{8.5}$	256	6.1
2	Sucrose density gradient centrifugation	$10^{8.1}$	4	7.5
	Gel chromatography	$10^{7.9}$	32	6.4

[1]Infectivity titer was determined by the method of Reed and Muench (1948) on SPF embryonated chicken eggs.
[2]The titer is expressed as the reciprocal of the highest sample dilution showing complete hemagglutination.
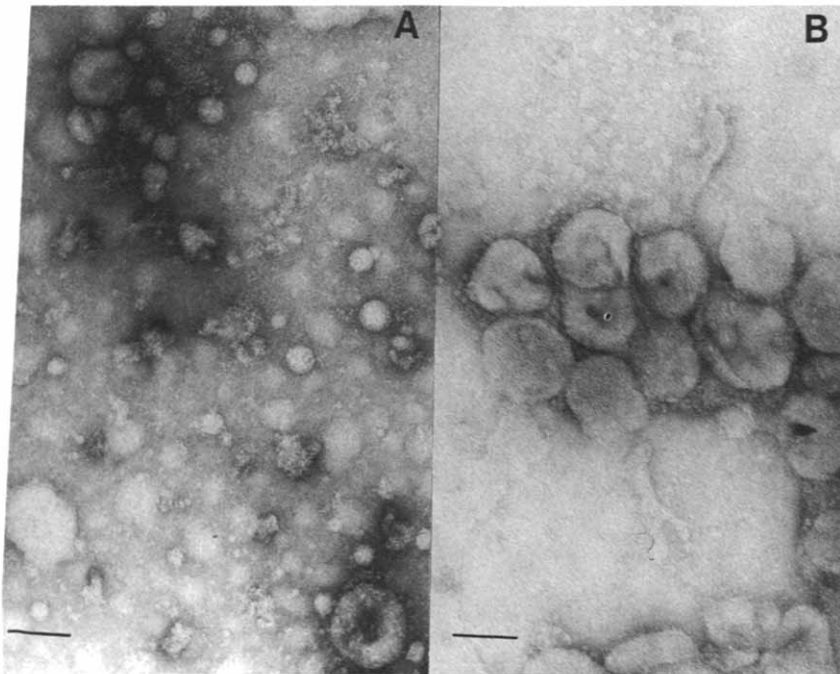

Fig. 2. Electron micrographs of IBV Strain M41. (A) Concentrated virus sample; (B) gel-chromatographically purified virus sample. Bar marker represents 100 nm.

Electron microscopy

Electron micrographs of IBV are shown in Fig. 2. Concentrated virus samples had much debris, whereas less debris was found in GC-purified virus samples. Furthermore, the spike retention rate of GC-purified virus samples was 60.2% and that of SDGC-purified virus was 22.8%.

Purity of virus preparation

SDS–PAGE of some virus preparations is shown in Fig. 3. Apparent virus structural proteins S1, S2, N, and M were observed in both GC- and SDGC-purified samples, but the amounts of S1 and S2 proteins in SDGC-purified samples were lower than those in the GC-purified samples.

The specific activity [$\log_{10}$(total infectivity titer)/(total protein)] of the purified virus samples was ~1000 times higher than that of the starting materials. Recovery of the GC-purified virus samples (17.8%) was lower than that of the SDGC-purified virus samples (23.4%) (Table 2).
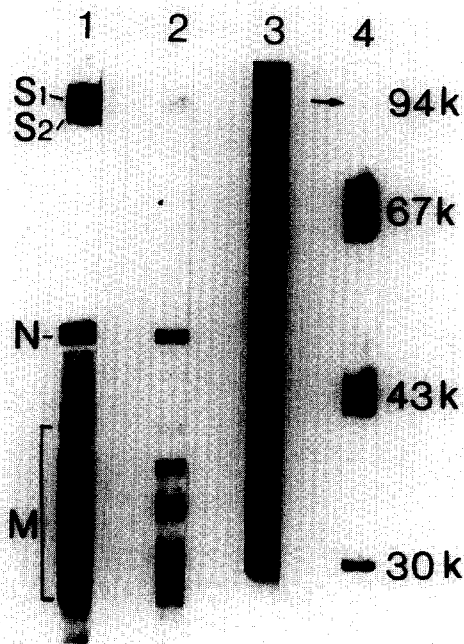

Fig. 3. SDS–PAGE of IBV Strain M41 was carried out as described in the text. Lanes: 1, purified virus sample by gel chromatography; 2, purified virus sample by sucrose density gradient centrifugation; 3, concentrated virus sample; 4, marker proteins. The numbers on the right-hand side show the molecular weights of marker proteins: phosphorylase "b", 94 kDa; albumin, 67 kDa; ovalbumin, 43 kDa; carbonic anhydrase, 39 kDa.

TABLE 2

Purification of IBV Strain M41 by two methods; sucrose density gradient centrifugation (SDGC) and gel chromatography (GC)

Step	Total volume (ml)	Total protein[1] (mg)	Total infectivity titer[2] ($\log_{10}$ EID$_{50}$)	Specific activity[3]	Recovery of infective virus (%)
Allantoic fluid	600	262.7	9.53	7.11	100
Concentrated virus[4]	2.5	6.68	9.15	8.32	41.7
SDGC	4.5	0.08	8.90	10.02	23.4
GC	6.0	0.05	8.78	10.08	17.8

[1]Protein concentration was determined by the method of Lowry et al. (1951).
[2]Infectivity titer was determined by the method of Reed and Muench (1948) on SPF embryonated chicken eggs. Total infectivity titer $= \log_{10}$ [total volume (ml) $\times$ EID$_{50}$ ml^{-1}].
[3]Specific activity $=$ total infectivity titer $- \log_{10}$ [total protein (mg)].
[4]Allantoic fluid was concentrated by centrifugation at 19 000 rpm (RPS 19 rotor, Hitachi, Japan) for 2 h.

DISCUSSION

Purification of IBV has generally been performed by SDGC. However, in some vaccine strains of IBV spike projections were easily released from virions during SDGC (Yagyu and Ohta, 1985). In addition, Stern and Sefton (1982) reported that S1 (GP90) was either degraded or released from extracellular virions by incubation of culture medium containing IBV (Strain B42) without cells overnight at 37°C, and they concluded that binding of S1 and S2 to virion was very weak. Therefore, purification by SDGC might cause dissociation of spike projections from the virion. An aim of the present study was to apply gel chromatography using a Sephacryl S-1000 column to purify some IBV strains in order to obtain intact purified virus samples from allantoic fluids.

HA units of GC-purified virus samples were ~10 times lower than those of SDGC-purified virus samples (Table 1). The result indicates that GC-purified virus samples possess more hemagglutinin on each virion. In addition, spike retention rate in electron microscopy of the GC-purified virus samples of Strain M41 was much higher than that of SDGC-purified virus samples. Furthermore, although a marked difference in hemagglutinating activity was observed between GC-purified and SDGC-purified virus samples, there was no difference to infectivity (Table 1). This result demonstrates that the polypeptide associated with HA is not directly involved in infectivity. Therefore, it seems that diminution of hemagglutinating activity in SDGC-purified virus samples did not result from steric hindrance of spike projections, but from decrease of the number of parts of those on the virions. Recently, Cavanagh and Davis (1986) reported that virions lacking S1 were no longer infectious to chick kid-

ney cells nor able to cause HA, whether or not they contained S2. However, such virions did retain the ability to attach to erythrocytes and cells. In contrast, both GC-purified and SDGC-purified virus samples showed infectivity to both embryonated chicken eggs (Tables 1 and 2) and chick kidney cells (data not shown). These results suggest that SDGC-purified virus samples retain a reduced amount of S1 on the surface of the virions, which is consistent with the result of SDS–PAGE (Fig. 3). Thus, it seems probable that we could obtain more spike-rich virions with high HA activity by GC purification than by SDGC.

Virus samples obtained by GC were checked for purity with the electron microscopy and SDS–PAGE. In SDS–PAGE, the M protein was not clearly separated (Fig. 3, Lane 1). It seems that there are some reasons for the indistinct separation of the M protein. The gels were stained by silver which is more highly sensitive than commonly used Coomassie blue. Furthermore, low amounts of contaminants with relatively low molecular weights from allantoic fluid may exist in the virus samples.

Specific activity of the purified virus was ~ 1000 times higher than that of the starting materials. There was no difference in the specific activity between GC-purified and SDGC-purified virus samples. These results indicate that GC using Sephacryl S-1000 column is a useful tool for purification of IBV.

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