

Purification of turkey coronavirus by Sephacryl size-exclusion chromatography

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Abstract

Sephacryl S-1000 size-exclusion chromatography was used to purify turkey coronavirus (TCoV) from infected turkey embryo. TCoV was propagated in the 22-day-old turkey embryos. Intestines and intestinal contents of infected embryos were harvested and homogenized. After low speed centrifugation, the supernatant was concentrated by ultracentrifugation through a cushion of 30 or 60% sucrose solution, or by ammonium sulfate precipitation. The purification methods included sucrose gradient and Sephacryl S-1000 size-exclusion chromatography. Ultracentrifugation through a cushion of 60% sucrose solution was better than the other two methods for concentration of TCoV from intestinal homogenate. The most effective method for purifying TCoV and removing extraneous materials was size-exclusion chromatography as analyzed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis. More spike-rich particles were observed in the sample purified by chromatography than those purified by sucrose gradient as examined by electron microscopy. Differentiation of turkey anti-TCoV antiserum from normal turkey serum was better achieved by ELISA plates coated with TCoV preparation purified by size-exclusion chromatography than that purified by sucrose density gradient. The results indicated that Sephacryl S-1000 chromatography was useful for purification of TCoV. © 2002 Elsevier Science B.V. All rights reserved.

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1. Introduction

Coronavirus is enveloped, positive-stranded RNA virus that possesses three major structural proteins: a predominant phosphorylated nucleocapsid (N) protein with molecular weight (Mr)

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ranging from 45 to 60 kd and two major virus encoded envelope proteins including peplomeric glycoprotein (spike protein, S) with Mr of 170–200 kd, that makes up the large surface projections of the virion and membrane protein (M) with an approximate Mr of 20–30 kd. Some coronaviruses have another envelop protein, hemagglutinin-esterase (HE), with a Mr ranging from 130 to 140 kd, that is a disulphide-linked

dimer of 65 kd subunits (Dea and Tijssen, 1988; Saif, 1993).

Turkey coronavirus (TCoV) is a member of the coronavirus genus. The diameter of TCoV virions varies from 50 to 150 nm. The viral particles bear the characteristic petal- or pear- shaped surface projections, giving it a morphologic appearance of a solar corona. The virus particle density ranges from 1.18 to 1.20 g/ml (Deshmukh and Pomeroy, 1974; Dea et al., 1985). Intact TCoV particles hemagglutinate rabbit, guinea pig (Dea et al., 1985), and rat erythrocyte (Dea and Tijssen, 1988).

Several studies have tried to grow TCoV in different primary cell cultures (Hofstad et al., 1969; Deshmukh et al., 1973; Dea et al., 1985). All these attempts were unsuccessful. The successful propagation of TCoV in a continuous cell line, HRT-18, was reported at 1989 (Dea and Tijssen, 1989). The HRT-18 cell is an established cell line derived from a human rectal adenocarcinoma (Tompkins et al., 1974). However, attempts to propagate the agent in HRT-18 cell in other laboratories (Guy et al., 1997; Ali and Reynolds, 1998) including ours have not been successful. Instead of cell culture, TCoV could be propagated by inoculating the amniotic cavity of embryonated turkey eggs older than 15 days (Adams and Hofstad, 1971). Intestines and intestinal contents obtained from infected turkey embryos were currently the only resource available for purification of TCoV.

Sucrose density gradient ultracentrifugation method is used frequently for virus concentration and purification. However, it was reported that the harsh force of ultracentrifugation might cause damage to virus, especially to enveloped virus. Alternatively, ammonium sulfate precipitation was suggested to be superior to ultracentrifugation for concentration of virus (Caul et al., 1978). Furthermore, size-exclusion chromatography was reported a better method than sucrose density gradient ultracentrifugation for purification of infectious bronchitis coronavirus (Nagano et al., 1989).

In this study, efforts were focused on the application of different methods for concentration and purification of the egg-propagated TCoV.

2. Materials and methods

2.1. Virus propagation

The TCoV Indiana isolate was obtained originally from field outbreak in southern Indiana. The agent was maintained in the laboratory by blind passages in turkey embryo. Intestines and intestinal contents collected from infected turkey embryos were homogenized in 5 volumes of PBS buffer, pH 7.4. After centrifugation of the homogenate at $3000 \times g$ for 10 min, the supernatant was used to inoculate amniotic sac of 22-day-old embryonated turkey eggs. Turkey embryos were incubated at 37 °C for 3 days. Intestines were harvested from inoculated turkey embryos. The harvested intestines were examined for the presence of TCoV by immunofluorescence staining. The harvested intestines of the same batch were used in concentration and purification by sucrose gradient ultracentrifugation or size-exclusion chromatography.

2.2. Immunofluorescent antibody staining

Frozen intestine was embedded in embedding medium and sectioned. Sections of 6 μm thick were obtained, air dried for 10 min, and fixed in acetone at room temperature for 10 min. Acetone fixed tissue sections were incubated at room temperature for 30 min with turkey anti-TCoV antiserum in a humidifying chamber. After washing with PBS buffer for 3 times, intestinal sections were incubated with fluorescein isothiocyanate-conjugated goat anti-turkey IgG (H + L) (Kirkegaard & Perry Laboratories, Gaithersburg, MD) at room temperature for 30 min in a humidifying chamber. Slides with intestinal sections were rinsed, air dried, and mounted. The slides were examined in a fluorescent microscope (Patel et al., 1975).

2.3. Concentration of virus

For concentration of virus, supernatants obtained from centrifugation of intestinal homogenate from infected turkey embryos were processed further and three different approaches

were tried and compared. Firstly, ultracentrifugation of the supernatant was carried out at $100\,000 \times g$ through a 15 ml cushion of 30% (w/v) sucrose solution in PBS buffer at 4 °C for 1 h. Secondly, ultracentrifugation of the supernatant was carried out at $100\,000 \times g$ through a 5 ml cushion of 60% (w/v) sucrose solution in PBS buffer at 4 °C for 1 h. Thirdly, supernatant was treated with 50% saturated ammonium sulfate and centrifuged at low speed ($8000 \times g$) at room temperature for 1 h. Because ultracentrifugation with a cushion of 60% sucrose solution was found to be better than the other 2 procedures after further examination, concentrated materials was referred to that obtained from this concentration procedure below.

2.4. Hemagglutination

Serial two-fold dilutions of viral antigen were prepared in PBS buffer and placed into 96-well V-bottomed microtiter plate in an amount of 50 μ l per well. Fifty microliters of 0.5% erythrocyte suspension of rabbit in PBS buffer was added into each well. The mixtures were then mixed, incubated at room temperature for 1 h, and the test was read (Dea et al., 1985). The hemagglutination (HA) titer was expressed as the reciprocal of the highest antigen dilution showing complete HA.

2.5. Density gradient ultracentrifugation

The concentrated materials were layered onto a 40–60% sucrose gradient with PBS buffer and ultracentrifuged at $100\,000 \times g$ for overnight. Fractions were collected from the bottom of the tubes and both absorbance and sucrose density of each fraction were determined. Virus-containing fractions were then collected (Dea and Tijssen, 1988). The first sucrose gradient prepared materials might be subjected further to one more time of repeated sucrose gradient ultracentrifugation.

2.6. Size-exclusion chromatography

Concentrated material containing TCoV was applied to a 2.5 by 95 cm Sephacryl S-1000 column (Bio-Rad, Hercules, CA) 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 0.5 ml/min. The OD 280 absorbance of eluents was monitored continuously. Every 5 ml of eluted material was collected. Fractions containing virus particles were pooled and may be concentrated by ultracentrifugation with a cushion of 60% (w/v) sucrose solution as described above.

2.7. Electron microscopy

Viral samples were ultracentrifuged at $100\,000 \times g$ for 2 h at 4 °C. Pellets were resuspended in distilled water and one drop of this suspension was placed on 200-mesh formvar carbon-coated grid. The grid was negatively stained with 2% of phosphotungstic acid, pH 6.5, and examined on electron microscope.

2.8. SDS-polyacrylamide gel electrophoresis and immunoblotting

The viral samples were solubilized in sample buffer containing 62.5 mM Tris-HCl, pH 6.8, 1% sodium dodecyl sulfate (SDS), 10% glycerol, 0.001% bromophenol blue, and 1% 2-mercaptoethanol and boiled for 5 min. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) was carried out using the discontinuous buffer system (Laemmli, 1970). Polypeptide bands were revealed by staining the gel with Coomassie brilliant blue G-250. For immunoblotting, viral polypeptides separated by SDS-PAGE were electrotransferred onto nitrocellulose membrane (Millipore, Bedford, MA) with transfer buffer containing 50 mM Tris, 384 mM glycine, and 20% (v/v) methanol, pH 8.3. Electrotransfer was carried out at 65 V for 1 h. The nitrocellulose membrane was incubated overnight in PBS containing 0.05% Tween 20 (PBS-T). After washing three times in PBS-T, membrane was incubated for 2 h at room temperature with turkey anti-TCoV antiserum at 1:500 dilution in PBS-T. Three times of washing was followed by addition of peroxidase-conjugated goat anti-turkey IgG (Kirkegaard & Perry Laboratories). After incubation of 2 h at room temperature, the membrane

was washed three times and covered with the peroxidase substrate, 3,3'-diaminobenzidine. The blot was allowed to develop and the reaction was stopped by washing the membrane in distilled water.

2.9. Enzyme-linked immunosorbent assay

Turkey coronavirus antigens purified by sucrose density gradient ultracentrifugation or size-exclusion chromatography were diluted with PBS buffer, coated on 96-well microtiter plates, and evaluated for capability to differentiate turkey anti-TCoV antiserum from normal turkey serum in enzyme-linked immunosorbent assay (ELISA). Serum positive for TCoV was the hyperimmune serum prepared from turkey infected experimentally with TCoV. Serum negative for TCoV was collected from a 4-month-old normal healthy turkey grown in isolation room in the laboratory. Dilutions of serum and conjugate were optimized by checkerboard tests. Serum samples were diluted in dilution buffer containing 150 mM phosphate buffer, 0.85% NaCl, 1% BSA, and 0.02% Tween-20 and 100 μ l of diluted serum sample was added to the well in duplicate. Plates were incubated at 37 °C for 1 h. After incubation, wells

were emptied and washed 3 times with PBS-T. Horseradish peroxidase-conjugated goat anti-turkey IgG (Kirkegaard & Perry Laboratories) diluted in dilution buffer was added to each well. Plates were incubated and washed as in the previous step, followed by the addition of 100 μ l of enzyme substrate, tetramethyl benzidine solution, to each well. After incubation at room temperature for 30 min, a 2 N HCl solution was added at 100 μ l/well. The absorbance value of each well was measured at 450 nm using a spectrophotometer (Vmax™ kinetic microplate reader, Molecular Devices Corporation, Menlo Park, CA). The absorbance values and ratios of positive control (PC) and negative control (NC) serum samples were calculated.

3. Results

3.1. Concentration of virus

In the ultracentrifugation through a cushion of 30% sucrose solution, TCoV were pelleted down to the bottom of the centrifuge tube. The recovery rate of HA activity in the pellet was 2.5% (Table 1, Procedure 1). In the ultracentrifugation

Table 1
Comparison of procedures for concentration of turkey coronavirus (TCoV)

| Procedures | Total volume (ml) | Total protein ^a (mg) | Total HA activity (unit) | Specific activity ^b (unit/mg) | Recovery ^c (%) |
|--|-------------------|---------------------------------|--------------------------|--|---------------------------|
| (1) 3000 \times g sup ^d | 20 | 156 | 64 000 | 410 | 100 |
| 30% cushion ppt ^e | 4 | 12 | 1600 | 133 | 2.5 |
| (2) 3000 \times g sup | 25 | 195 | 80 000 | 410 | 100 |
| 60% cushion band ^f | 4 | 63 | 53 760 | 853 | 67.2 |
| (3) 3000 \times g sup | 10 | 78 | 6400 | 82 | 100 |
| (NH ₄) ₂ SO ₄ ppt ^g | 2.3 | 25 | 1472 | 60 | 23.0 |

^a Protein concentration was determined by the method of Lowry et al. (1951).

^b Specific activity = total HA activity/total protein.

^c Recovery = total HA activity of d or e/total HA activity of d.

^d Supernatants from centrifugation of infected turkey embryo intestinal homogenates at 3000 \times g for 10 min.

^e Precipitates from ultracentrifugation of 3000 \times g sup at 100 000 \times g through a 15 ml cushion of 30% (w/v) sucrose solution for 1 h.

^f Opalescent band from ultracentrifugation of 3000 \times g sup at 100 000 \times g through a 5 ml cushion of 60% (w/v) sucrose solution for 1 h.

^g Precipitates from treatment of 3000 \times g sup with 50% saturated ammonium sulfate and low speed centrifugation (8000 \times g).

Table 2

Purification of turkey coronavirus (TCoV) by ultracentrifugation of concentrated material through two times of continuous 40–60% (w/v) sucrose gradient

| Step | Total volume (ml) | Total protein ^a (mg) | Total HA activity (unit) | Specific activity ^b (unit/mg) | Recovery ^c (%) | Purification factor ^d |
|-------------------------------|-------------------|---------------------------------|--------------------------|--|---------------------------|----------------------------------|
| 3000 × g sup ^e | 217 | 2170 | 34 720 | 16 | 100.0 | 1.0 |
| 60% cushion band ^f | 17 | 99 | 21 760 | 221 | 62.7 | 13.8 |
| First gradient ^g | 16 | 16 | 5216 | 320 | 15.0 | 20.0 |
| Second gradient ^h | 20 | 7 | 3260 | 476 | 9.4 | 29.8 |

^a Protein concentration was determined by the method of Lowry et al. (1951).

^b Specific activity = total HA activity/total protein.

^c Recovery = total HA activity of e, f, g, or h/total HA activity of e.

^d Purification factor = specific activity of e, f, g, or h/specific activity of e.

^e Supernatants from centrifugation of infected turkey embryo intestinal homogenates at 3000 × g for 10 min.

^f Opalescent band from ultracentrifugation of e at 100 000 × g through a 5 ml cushion of 60% (w/v) sucrose solution for 1 h.

^g Opalescent band from ultracentrifugation of f at 100 000 × g through a continuous 40–60% (w/v) sucrose gradient for 16 h.

^h Opalescent band from two times of ultracentrifugation of f at 100 000 × g through continuous 40–60% (w/v) sucrose gradient for 16 h.

through a cushion of 60% sucrose solution, viral particles were banded on top of the sucrose solution. The HA activity recovered in the opalescent band was 67.2% (Table 1, Procedure 2). Treatment with 50% saturated ammonium sulfate and centrifugation at lower speed (8000 × g) recovered 23.0% HA activity in the pellet containing TCoV (Table 1, Procedure 3).

3.2. Density gradient ultracentrifugation

In the sucrose gradient, two absorbance peaks corresponding to densities of 1.18–1.20 and 1.14–1.15 g/ml, respectively, were observed. According to examination by electron microscopy, the fractions of 1.18–1.20 g/ml contained more intact TCoV particles with characterized petal- or club-shaped surface projections. Fractions of both peaks had infectivity to 22-day-old turkey embryos via the amniotic cavity route of inoculation. One or two times of continuous 40–60% sucrose gradient ultracentrifugation were carried out and compared for purification of TCoV. After the first sucrose gradient ultracentrifugation, the recovery of HA activity was decreased from 62.7% (with a 60% sucrose cushion) to 15.0% while the purification factor was increased from 13.8 to 20.0 (Table 2). After the second sucrose gradient ultrac-

trifugation, the recovery of HA activity was further decreased to 9.4% while the purification factor was improved to 29.8% (Table 2). Analysis of proteins from purified material by SDS-PAGE did not reveal obvious difference in protein patterns and intensities between the preparation of the first sucrose gradient ultracentrifugation (Fig. 2; Lane 4) and that of the second consecutive continuous sucrose gradient ultracentrifugation (Fig. 2; Lane 5). The protein patterns of these sucrose gradient preparations (Fig. 2; Lanes 4 and 5) were similar to that of the concentrated materials from ultracentrifugation through a cushion of 60% sucrose solution (Fig. 2; Lane 3). The protein patterns that were recognized by turkey anti-TCoV antiserum in the immunoblotting analysis were also very similar among these sucrose gradient preparations and the concentrated materials (Fig. 3; Lanes 3, 4, and 5).

3.3. Size-exclusion chromatography

Two clearly separated peaks of OD 280 were observed in the elution profile of size-exclusion chromatography (Fig. 1). The first peak was small. The second peak was large and comprised at least two different sizes of proteins that could not be separated. Coronavirus particles were

found only in the first peak, as revealed by electron microscopy. It appears that more contaminant proteins were removed by size-exclusion chromatography than by sucrose gradient ultracentrifugation when the protein contents of the first peak fractions (Fig. 2; Lane 6) or the sucrose gradient preparation (Fig. 2; Lane 4) were compared to that of the concentrated material (Fig. 2; Lane 3). The protein pattern of the second peak (Fig. 2; Lane 7) was very similar to that of the original supernatant of intestinal homogenate (Fig. 2; Lane 2). The major protein bands of the first peak (Fig. 2; Lane 6) could be grouped into 200 kd, 90–100 kd, 60–70 kd, 50 kd, and 25–35 kd. Furthermore, most of these major protein bands were recognized by turkey anti-TCoV antisera in the immunoblotting analysis (Fig. 3; Lane 6). In contrast, most of the protein bands of the second peak (Fig. 3; Lane 7), like those of the original supernatant of intestinal homogenate (Fig. 3; Lane 2), were not recognized by turkey anti-TCoV antisera.

3.4. ELISA

As shown in Fig. 4, the ratio of PC/NC using TCoV antigen purified by sucrose density gradient

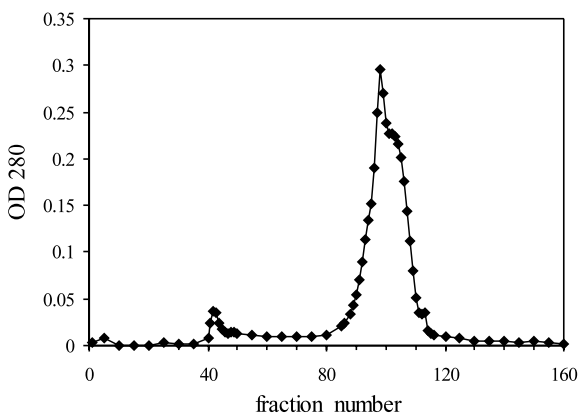


Fig. 1. Elution profile of size-exclusion chromatography with a Sephacryl S-1000 column ($2.5 \times 95 \text{ cm}^2$) of turkey coronavirus Indiana isolate. The column was eluted at a flow rate of 0.5 ml/min with 0.02 M phosphate buffer (pH 7.2) containing 0.15 M NaCl. The fraction size was 5 ml. The absorbance of OD 280 of each fraction was determined. Turkey coronavirus particles were seen in only fractions of the first small peak.

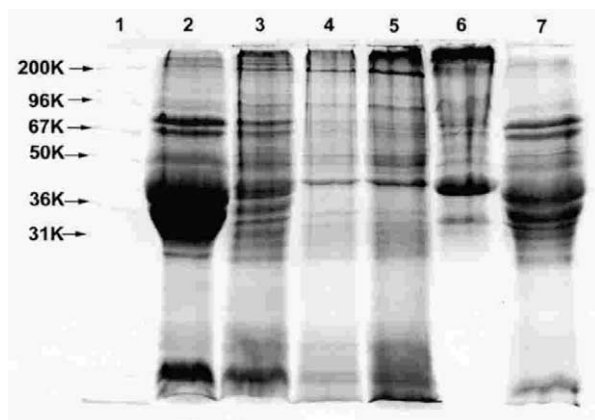


Fig. 2. SDS-PAGE analysis of protein contents of purified materials. Purified preparations were electrophoresed in 10% gel under reducing conditions. Lanes 1, Mr markers; lane 2, supernatants from centrifugation of infected turkey embryo intestinal homogenates at $3000 \times g$ for 10 min; lane 3, opalescent band from ultracentrifugation of $100\,000 \times g$ through a 5 ml cushion of 60% (w/v) sucrose solution for 1 h; lane 4, opalescent band from ultracentrifugation of $100\,000 \times g$ through a continuous 40–60% (w/v) sucrose gradient for 16 h; lane 5, opalescent band from two consecutive ultracentrifugation of $100\,000 \times g$ through a continuous 40–60% (w/v) sucrose gradient for 16 h; lane 6, the first small peak of the Sephacryl S-1000 size-exclusion chromatography; lane 7, the second large peak of the Sephacryl S-1000 size-exclusion chromatography.

ultracentrifugation as coating antigen was lower than that using TCoV antigen purified by size-exclusion chromatography as coating antigen. The PC/NC ratios were less than 6 throughout the different concentrations of sucrose gradient-purified TCoV antigen tested. The PC/NC ratios varied among the different concentrations of size-exclusion chromatography-purified TCoV antigen tested and the maximum ratio about 20 was observed when the coating concentration was 10 $\mu\text{g/ml}$.

4. Discussion

The destructive effect of ultracentrifugation to viral particles had been noted (Caul et al., 1978). It was generally thought helpful to alleviate the harsh force of ultracentrifugation by a cushion of sucrose solution. However, only 2.5% of HA ac-

tivity was recovered after ultracentrifugation through a cushion of 30% sucrose solution. In contrast, the recovery of HA activity by ultracentrifugation through a cushion of 60% sucrose solution when the viral particles banded on top of the sucrose solution and did not reach the bottom of the centrifuge tube was more than 20 times higher than that of ultracentrifugation through a cushion of 30% sucrose solution. These results suggest that the deleterious effect to the viral particles might occur when the viral particles reached the bottom of the centrifuge tube during ultracentrifugation. The low recovery rate (23.0%) of HA activity by ammonium sulfate treatment was unexpected. It was reported that ammonium sulfate precipitation was better than the ultracentrifugation method for routine examination of viruses in faecal specimens by electron microscopy (Caul et al., 1978). Although saturated ammonium sulfate and low speed centrifugation could

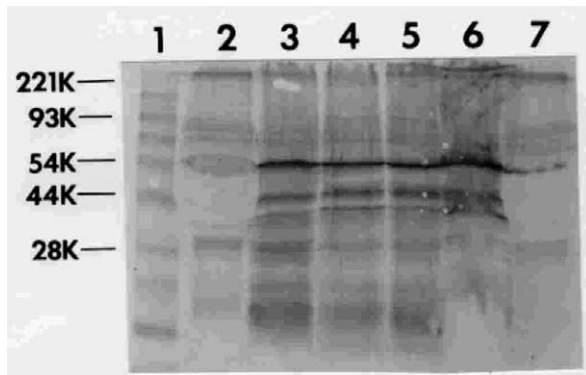


Fig. 3. Immunoblotting analysis of protein contents of purified materials with turkey anti-TCoV antiserum. Purified preparations were electrophoresed, transferred onto nitrocellulose membrane, and reacted with anti-TCV antiserum. Lanes 1, Mr markers; lane 2, supernatants from centrifugation of infected turkey embryo intestinal homogenates at $3000 \times g$ for 10 min; lane 3, opalescent band from ultracentrifugation of $100\,000 \times g$ through a 5 ml cushion of 60% (w/v) sucrose solution for 1 h; lane 4, opalescent band from ultracentrifugation of $100\,000 \times g$ through a continuous 40–60% (w/v) sucrose gradient for 16 h; lane 5, opalescent band from two consecutive ultracentrifugation of $100\,000 \times g$ through a continuous 40–60% (w/v) sucrose gradient for 16 h; lane 6, the first small peak of the Sephacryl S-1000 size-exclusion chromatography; lane 7, the second large peak of the Sephacryl S-1000 size-exclusion chromatography.

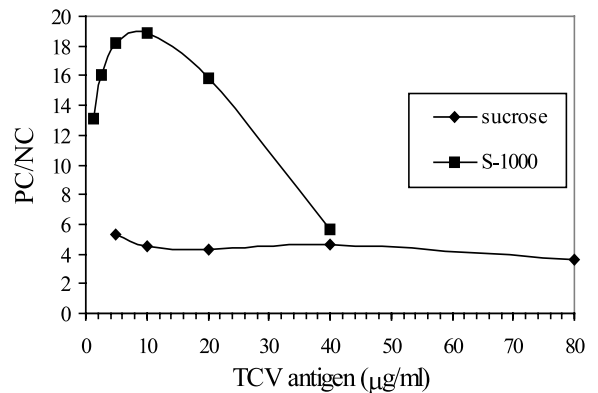


Fig. 4. Comparison of ELISA plates coated with turkey coronavirus (TCoV) preparations purified by sucrose density gradient or Sephacryl S-1000 size-exclusion chromatography for differentiation between turkey anti-TCoV antiserum (PC) and normal turkey serum (NC). The PC/NC is the ratio of anti-TCoV antiserum absorbance value to normal serum absorbance value.

avoid the deleterious effect of harsh force due to ultracentrifugation on TCoV, high concentrated salt might have resulted in adverse hypertonic effect to the envelope of virus particles and damage to the viral surface structure. Therefore, concentration of TCoV from the supernatants after centrifugation of infected turkey embryo intestinal homogenate was better achieved by ultracentrifugation of the supernatants at $100\,000 \times g$ through a cushion of 60% sucrose solution.

Sucrose density gradient ultracentrifugation is used commonly for purification of viruses (Naqi et al., 1975). However, the sucrose gradient ultracentrifugation maybe not a good way for purification of TCoV based on the observations that coronavirus particles were found in fractions collected from either major peaks, fractions between the two major peaks, and also fractions below the higher density peak. In addition, the protein contents were similar between sucrose gradient preparations and the concentrated materials as revealed in SDS-PAGE and immunoblotting.

For purification of another avian coronavirus, infectious bronchitis virus, it was reported that Sephacryl S-1000 size-exclusion chromatography was better than sucrose density gradient ultracentrifugation (Nagano et al., 1989). In line with this

result, the Sephacryl S-1000 size-exclusion chromatography was found superior to the sucrose density gradient ultracentrifugation for the purification of TCoV in the present study. By size-exclusion chromatography, coronaviral particles were observed only in the first small peak fractions but not in the late large peak fractions, indicating that a lot of extraneous materials in the intestinal contents were separated from the TCoV particles. This was confirmed further by the results of SDS-PAGE and immunoblotting that the second peak fractions contained many protein bands that shared similar pattern with the original supernatant of intestinal homogenate. In addition, most of these proteins were not detectable in the immunoblotting analysis. Comparatively, most of the protein bands of the first peak were recognized by turkey anti-TCoV antiserum in the immunoblotting analysis.

According to electron microscopy, the size-exclusion chromatography preparation contained less debris while more intact coronavirus particles than the sucrose gradient preparation. The size-exclusion chromatography preparation was better than the sucrose gradient preparation to be used as coating antigen in the ELISA for discrimination between turkey anti-TCoV antiserum and normal turkey serum. These results indicated that Sephacryl S-1000 size-exclusion column chromatography was a useful method for purification of TCoV.

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