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Purification of avian influenza virus (H9N2) from allantoic fluid by size-exclusion chromatography

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Abstract: Avian influenza viruses represent a rising threat of pandemic influenza. Allantoic fluid including influenza virus H9N2 was run on downstream processing to purify the virus. To do this, allantoic fluid was clarified and concentrated. It was applied to size exclusion chromatography. Different parameters such as ovalbumin, protein content, and hemagglutinin activity were determined to confirm the purity. The results may suggest that this method is quite efficient for achieving a purified H9N2 influenza virus and further reveal that size-exclusion chromatography is a practical method for purifying avian influenza viruses. In conclusion, this procedure can be used in vaccine production and antiserum preparation as an alternative to traditional methods and also can be considered in the purifying of other viruses when there is no facility of ultracentrifuge or zonal centrifuge.

Key words: Avian H9N2 influenza virus, chromatography, vaccine

1. Introduction

Avian influenza (AI) refers to flu caused by type A strains of the influenza virus that is an infectious disease of birds. Influenza viruses are classified into three types: A, B, and C. Only influenza A viruses infects birds. The avian influenza virus (AIV) is a member of the family *Orthomyxoviridae*, containing negative-sense and single-stranded RNA (1). Based on their pathogenicity, two types of AIV have been described: the highly pathogenic type (HPAIV), which causes severe disease with high mortality, and the low pathogenic type (LPAIV), inducing asymptomatic or mild infection (2).

According to the main surface proteins, hemagglutinin (HA) and neuraminidase (NA), influenza A viruses are categorized into different subtypes: 17 for HA and 10 for NA. The HA of the influenza virus is the major surface protein inducing protective immune responses (3). According to the World Health Organization (WHO), all H9 viruses found in wild birds and poultry are LPAIVs.

During 1994–1999, infections in poultry that were caused by influenza viruses of the H9 subtype were noticeably prevalent. Outbreaks of the H9N2 subtype occurred in domestic ducks, chickens, and turkeys in Europe and the United States (4). This type of bird flu was reported in 1998 on a broiler chicken farm in Tehran, Iran, and from neighboring provinces (5,6). Vaccines have an important role in controlling and protecting the poultry industry from infectious diseases. Influenza vaccines are usually prepared from a mix of an adjuvant and harvested allantoic fluid. The resulting product includes a high amount of unwanted impurities of allantoic fluid such as ovalbumin. For this reason, applying purification procedures in vaccine production is important.

Generally, viruses have been purified by a variety of methods, including PEG/NaCl precipitation (7), ultracentrifugation (8), column chromatography (9), high-performance liquid chromatography (10), and centrifugation on a sucrose gradient, which are mostly used on the laboratory scale. In 1977, centrifugation on a sucrose gradient was carried out for avian influenza purification (11). Chromatography has been suggested as a novel method in virus purification (9). Here we describe a standard strategy for the purification of avian influenza virus using centrifugation, ultrafiltration, and chromatography techniques.

In this study, we report a simple and effective procedure for concentrating and purifying the avian influenza H9N2 virus with size-exclusion chromatography. Our goal was to set up a platform for purification of the virus that can be applied from small to large scale in vaccine production and antiserum preparation instead of other techniques such as zonal centrifugation.

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2. Materials and methods

2.1. Egg incubation and antigen preparation

Avian influenza virus strain A/chicken/Iran/259/1998 (H9N2), confirmed by the Central Veterinary Laboratory of Webridge, Surrey, UK, was propagated in 10-day-old specific pathogen-free embryonated chicken eggs (12). After incubation for 48 h at 37 °C, the infectious allantoic fluid was harvested and pooled. The fresh allantoic fluid was clarified and host debris was separated from it by centrifuging at 4500 rpm for 60 min at 4 °C, and then the clarified fluid was checked for its HA titer (13). Clarified fluid was inactivated by formalin at 1/2000 for 2 days at 37 °C. Three batches of each active and inactive virus were used in the next steps of this work in three different conditions.

2.2. Ultrafiltration of the virus

After clarifying active and inactive antigens, they were concentrated 10-, 20-, and 35-fold using cross-flow ultrafiltration through a 100-kDa cutoff cassette (Slice 200, Sartorius, Germany) at room temperature. The permeated and concentrated samples were gathered in intervals to determine the HA activity. The concentration process was repeated twice for each batch. Phosphate-buffered saline (PBS), 10 mM, and 50 mM NaCl, pH 7.5, were used in all steps for washing and diafiltration. All of the concentrated samples were kept at –70 °C for future experiments.

2.3. Purification of the virus

Size-exclusion chromatography of concentrated samples containing both active and inactive influenza virion was performed separately using Sephadex G-75 and G-100 and Sepharose CL-4B (GE Healthcare) resins in PBS at room temperature. Each sample (10-, 20-, and 35-fold concentrated allantoic fluid) was loaded on a column of 100 cm × 2.5 cm, which was packed with the above resins and equilibrated with the same buffer. To separate the virus, different amounts of concentrated sample (2%–10% of the total bed volume) were loaded into the column and washed at a flow rate of 0.5–1 mL/min. All fractions were collected and analyzed for their absorbance at 260 nm and 280 nm using a UV/VIS spectrophotometer (Pharmacia Biotech, Ultraspect 2000), and for HA activity, ovalbumin, and protein concentration (Bradford assay (14), Bio-Rad kit, USA).

2.4. HA titer test

HA assay was performed to assess the viral titration in all steps. To do this, 50 µL of PBS was added to a round-bottomed 96-well plate (Nunc, Pasadena, TX, USA) and an equivalent volume of virus sample was added to the first rows and mixed well. Then 50 µL of the previous suspension was transferred to the next following well. Twofold serial dilutions of 50 µL of virus suspension were made across the plate. A volume of 1% chicken's red blood cells was added to dilute virus suspensions. The microplate was kept at room temperature for 30 min. The red blood cells that were not bound by influenza virus sunk to the bottom of the well, defining the HA activity (13).

2.5. Protein assay

Protein concentration was determined by quick-start Bradford protein assay (Bio-Rad). The standard curve was prepared using a range of dilutions of bovine serum albumin according to instructions of the manufacturer. All samples were measured in duplicate.

2.6. SDS-PAGE

Proteins in the processed fractions were visualized using sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) based on the Laemmli method (15). The purified viral samples from active and inactive batches were separated on 12% polyacrylamide gel. Gel electrophoresis (SDS-PAGE) was carried out using the Mini-PROTEAN Bio-Rad system. Polypeptide bonds were revealed by staining the gel with silver nitrate.

2.7. Ovalbumin ELISA test

Indirect enzyme-linked immunosorbent assay (ELISA) was used to detect ovalbumin in allantoic fluid and purified samples. The test was carried out based on the supplier's protocol (Seramun Diagnostica GmbH, Heidesee, Germany) for different dilutions.

3. Results

3.1. Harvesting and concentrations

A total of 6 batches of inactive and active allantoic fluid including H9N2 virus were produced and clarified and checked for their HA activity. HA activity was determined in all concentrated samples, including influenza viruses. These samples revealed no loss of HA activity before and after this step. The results from the HA test of influents and effluents of each sample showed no virus penetration during cross-flow filtration, while the HA activity in concentrated samples was correlated with the fold of concentration in both active and inactive viruses (Table). HA activity in both active and inactive concentrated samples showed a constant stability based on this test after more than 6 months at 4 °C.

3.2. Purification

In this study two forms of viruses were applied in a varied range of gel filtration resins to find out the efficiency of column chromatography in virus purification. In general, all columns were run with the same buffer and same conductivity, whereas the volume of the loaded sample and the flow rate were variable. Neither Sephadex G-75 nor G-100 acted as a useful resin for separation of the influenza virus from other allantoic fluid proteins (data not shown). However, the Sepharose CL-4B column was capable of separating the influenza H9N2 virus from

Table. Specification of viral samples through the purification procedure.

Samples	HA titer [a]	Protein, mg/mL	Ovalbumin, µg/mL
Active H9N2 virus (concentrated) [b]	15	16	82
Purified active H9N2 virus	13	0.66	0.13
Inactive H9N2 virus (concentrated) [b]	13	18	68
Purified inactive H9N2 virus	12	0.4	0.07

[a] HA titer in all permeated samples was zero.
[b] The concentration factor for this sample was equal to 20.

other proteins of the allantoic fluid. The volume of the loaded sample from different folds of concentrations in both active and inactive forms was used to optimize the column's parameters. Comparing all acquired data showed the best virus purification with a flow rate of 40–60 mL/h and sample volume of up to a maximum of 10% of the total bed volume. Figure 1 illustrates the behavior of the Sepharose CL-4B column loaded with 2% of the void volume with the active H9N2 virus. Comparing HA activity along with absorbance of fractions at 260 and 280 nm showed complete separation of viral particles in the first peak. In the next step, the Sepharose CL-4B column was loaded with 10% of the void volume with the inactivated H9N2 virus. Based on this chromatogram, only the eluted fractions from the first peak showed HA activity (Figure 2).

The amount of ovalbumin as the main impurity in allantoic fluid was determined by ELISA assay to find out the purity of the virus. The contents of ovalbumin in concentrated and permeated samples were over the range of kit limitations, while in pure samples it was less than

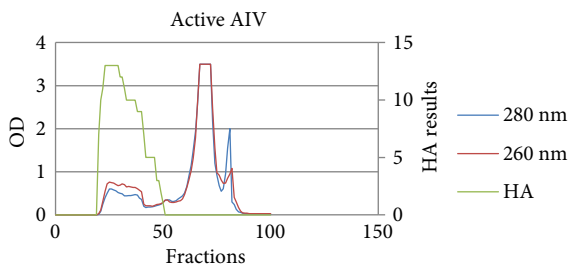

Figure 1. Chromatography of active concentrate of H9N2 virus on Sepharose CL-4B loaded with 2% of bed volume. UV absorbance at 280 nm (blue line), UV absorbance at 260 nm (red line), and HA activity (green line) are illustrated.

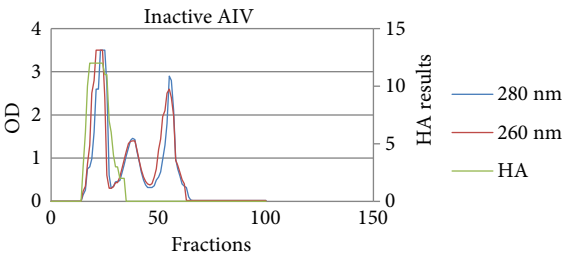

Figure 2. Chromatography of inactive concentrate of H9N2 virus on Sepharose CL-4B loaded with 10% of bed volume. UV absorbance at 280 nm (blue line), UV absorbance at 260 nm (red line), and HA activity (green line) are illustrated.

0.2 µg/mL, within the range of WHO limitations for egg-based human influenza vaccine (Table).

SDS-PAGE analysis of purified active and inactive samples showed that the purified virus was in agreement with the findings of a previous study (16). Although the same concentrations were used for both active and inactive samples, the bands in the inactive sample were observed with lower intensity, probably because of having formalin (Figure 3).

4. Discussion

The crucial role of influenza virus in veterinary and human fields forced researchers and industries to prepare the whole virus particle through different purifying methods. For instance, influenza virus has been separated on a sucrose gradient in the laboratory and on a large scale (11,17). Downstream processing in vaccine production has a great role and is a crucial step. Most of the efforts in virus purification recently moved towards column chromatography, such as size-exclusion chromatography (18), which might be exploited as the final step.

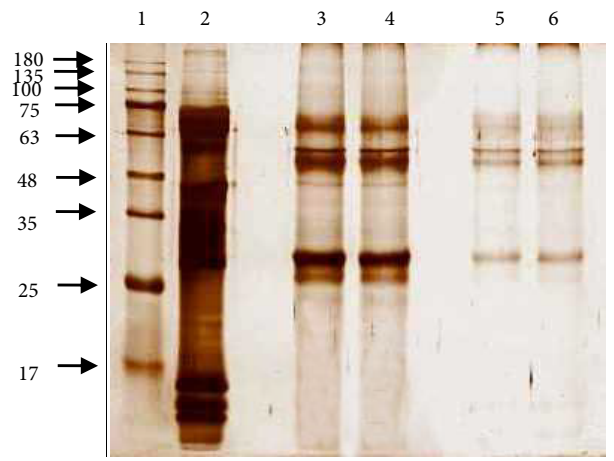

Figure 3. SDS-PAGE of purified sample. Lane 1, protein marker; lane 2, harvested allantoic fluid; lane 3, active AIV purified from first batch; lane 4, active AIV purified from 4th batch; lane 5, inactive AIV purified from first batch; lane 6, inactive AIV purified from 4th batch.

Clarified egg-derived avian influenza H9N2 was concentrated through tangential cross-flow filtration using a 100-kDa cassette a maximum of 30 times that was fed with active and inactivated virus. A similar method was reported using cell-derived human influenza virus with high recovery of HA activity, as resulted in this work (9). This step was carried out for different volumes of samples from 1 to 10 L. In all conditions, the permeated samples did not show any detectable HA activity (Table), while HA activity was increased in the concentrated part. By increasing the concentration factor and volume of samples, data showed that the filtration step of this method is effective to use in large-scale concentration of the virus with high HA activity before column chromatography.

The avian H9N2 subtype was purified at laboratory scale after separation by a sucrose gradient at a sufficient amount to use in laboratory analysis tests (13). Compared to that, here we set up column chromatography for large-scale purification of avian influenza. Among the resins that were used in this study, Sepharose Cl-4B showed the best separation. This column was able to separate whole intact viral particles from ovalbumin as the major protein of the allantoic fluid in the first peak. As shown in Figures 1 and 2 for both active and inactive forms of sample, the behavior of separation is nearly identical, with complete separation of ovalbumin. In addition, because of the large size of influenza virions, the mass transfer effects may enhance tailing of the elution peak (Figure 1). Compared to other studies on different viruses such as turkey coronavirus (19) and equine influenza (20), whole virus particles were recovered in the void volume.

This study presented a straightforward downstream process that can be carried out in the purification of active and inactive avian H9N2 influenza virus. All operations will be scaled up for application as a method in vaccine production and this may be useful in the concentration of other enveloped viruses.

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