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Downstream processing of Vero cell-derived human influenza A virus (H1N1) grown in serum-free medium

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ABSTRACT

A downstream processing was examined for Vero cell-derived human influenza virus (H1N1) grown in serum free medium. Vero cell banks were established in serum free medium and characterized according to regulatory requirements. Serum free Vero cells were grown on Cytodex 3 microcarriers in 5 L bioreactor and infected with influenza A virus (A/New Caledonia/99/55). The harvests were processed with the sequence of inactivation, clarification, anion exchange chromatography (DEAE FF), Cellufine Sulfate Chromatography (CSC) and size exclusion chromatography (Sepharose 6FF). Host cell DNA (hcDNA) was mainly removed with DEAE FF column and CSC by 40 and 223 fold, respectively. Most of Vero cell proteins were eliminated in CSC and Sepharose 6FF unit operation by about 13 fold. The overall scheme resulted in high recovery of hemagglutinin (HA) activity and the substantial removal of total protein, host protein and DNA. The total protein content and DNA content per 15 μg HA protein in final product was 89 μg and 33 pg, respectively, which complied with regulatory requirements for single strain influenza vaccines. SDS-PAGE analysis and Western blotting confirmed the purity of the final product. In conclusion, the suggested downstream process is suitable for the purification of microcarrier-based cell-derived influenza vaccine.

1. Introduction

Influenza virus, a lipid-enveloped RNA virus from the *Orthomyxoviridae* family, affects up to 10% of the world population annually. Five influenza pandemics have occurred in 1918, 1957, 1968, 1977 and the recent 2009 pandemic since last century. Large-scale vaccination remains the top strategy to control the spread of influenza infection and reduce the impact on public health. Conventionally, influenza vaccines are supplied by embryonated egg-based technology. The supply of embryonated eggs is available only while advanced planning and may be limited in the event of an influenza pandemic caused by avian influenza viruses. Hence, the embryonated egg-based technology may not be adequate to meet the rapid, immediate and surge needs for influenza vaccines in the event of an influenza pandemic. Based on these, WHO recommended to develop cell culture as an alternative substrate for the production of influenza vaccines in 1995 [1]. Currently, three continuous cell lines, Madin Darby canine kidney (MDCK) cells, African green monkey kidney Vero cells and the human retina-derived cell line Per. C6 have been investigated as candidate substrate for influenza vaccine production. Vero cells have been extensively used in the manufacture of human vaccine production, such as rabies vaccines and polio vaccines. Vero cell line has been used as host for the production of seasonal influenza vaccine [2] and H5N1 vaccine for pandemic flu [3]. Although influenza viruses were able to effectively propagate in Vero cells, the titres were relatively lower than that in embryonated eggs [2]. Therefore, it is of great significance to establish an efficient processing to increase the yield of cell culture-derived influenza vaccine.

Downstream processing of influenza virus from egg-derived influenza virus usually consists of clarification followed by ultrafiltration and sucrose zonal gradient centrifugation [4,5]. This process has been successfully employed for the production of influenza vaccine in Vero cells [2]. The greatest shortage in this downstream processing is lack of purification steps to effectively remove host cell DNA (hcDNA) and proteins. Recently, chromatography technology has been applied to purify cell culture-derived influenza vaccines, including size exclusion chromatography (SEC) [6,7], anion exchange chromatography (AEC) [7,8] and affinity chromatography [9,10]. A combination of SEC and AEC was examined to purify MDCK cell-derived influenza virus [7].

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Nevertheless, the final product failed to fulfill the purity requirements for protein and hcDNA. Thus, a combination of different downstream processing methods is needed to reduce these impurities.

Due to the potential of cellular transformation [11] the elimination of host cell genome is one of the major concerns for influenza vaccines produced in continuous cell lines. Efforts to eliminate hcDNA centered on enzymatic digestion [2,12], selective DNA precipitation [13,14], AEC [15] and recently hydrophobic interaction chromatography [16]. Enzymatic digestion and selective DNA precipitation may necessitate an additional purification or assay to confirm the complete removal of nuclease and precipitant reagents. AEC was widely applied to reduce hcDNA contamination in monoclonal antibody production [17–19], gene therapy vector [12,20,21] and vaccines [7,22,23]. Weak anion exchanger DEAE resin exhibited low adsorption capacity to virus and has been successfully utilized for the production of rabies vaccine [22,23]. In this study, DEAE chromatography was explored to remove hcDNA in Vero cell-derived influenza vaccine.

Immuno-chromatography, which captured influenza virus using polyclonal [24] or monoclonal antibodies [10], is costly and difficult for scaling up. Hemagglutinin (HA) is a glycoprotein containing several *N*-linked glycosylationsides. These glycans can be targeted as affinity ligands by specific lectins. Lectin affinity was reported to result in high HA activity recovery as well as substantial removal of total protein and hcDNA from cell culture-derived influenza broth [9,25]. Lectin affinity chromatography is not an optimal option due to potential toxicity of lectins. Compared to lectin affinity chromatography, pseudo-affinity chromatography offers a cheaper and easier alternative to capture influenza viruses. Cellufine Sulfate media, which contains sulfated cellulose beads and mimics the affinity of heparin or dextran sulfate has been used for the purification of many viral vaccines including influenza vaccine [26,27], Japanese encephalitis vaccine [28] and herpes simplex virus vaccine [29].

A typical influenza virus consists of about 500 molecules of HA (72 kDa) and 100 molecules of neuraminidase (42 kDa). In contrast, the molecular weight of most soluble proteins is not larger than a few hundred kDa. Several SEC media have been explored for the separation of influenza virus particles [6,7,30,31]. Efficient separation of influenza virus from cell components was achieved with Sepharose CL-2B, 4FF and 6FF [7]. Compared to Sepharose CL-2B and 4FF, Sepharose 6FF is more rigid and featured with higher flow rate. Hence, Sepharose 6FF was chosen as SEC media in this study.

The differences in starting materials, i.e., allontoic fluids versus cell culture media with microcarriers and cell debris, necessitate more complicated purification process for cell culture-derived influenza vaccine compared to egg-based influenza vaccines. Unfortunately, very few complete downstream processes have been described in details. In this study, we presented a complete purification process for Vero cell-derived influenza vaccine. First, we established Vero cell banks in serum free medium and tested for potential contamination, adventitious viruses and tumorigenicity according to regulatory requirements. Vero cells were used to propagate influenza virus on microcarriers in 5 L bioreactor. After harvesting, virus broth was purified with a sequence of inactivation, clarification, AEC anion exchange chromatography, Cellufine Sulfate Chromatography (CSC) and SEC. HA activity, which reflects the activity of viral particles to agglutinate erythrocytes, was used to evaluate the performance of a downstream process for influenza vaccine purification. This novel scheme resulted in high HA recovery and the efficient reduction of impurities. The content of total protein and hcDNA in the final inactivated influenza vaccine complied with the regulatory requirements.

2. Materials and methods

2.1. Cell culture and virus culture

Vero cells (Vero CCL-81) at passage 120 were obtained from ATCC and adapted to serum free growth using direct adaptation and serial passages for more than 6 passages in serum free medium VP-SFM (Invitrogen, USA). Master Cell Bank (MCB) and Working Cell Bank (WCB) were established in VP-SFM at passage 133 and 137, respectively. MCB and WCB have been fully characterized for adventitious agents according to Chinese Pharmacopeia [32] and fulfilled all the requirements for biological products. A single ampoule of WCB cells was thawed and passaged as monolayers in 150 cm^2 or 4 L rolling bottles to produce enough cells to inoculate a 5 L fermenter (New Brunswick Scientific, USA) using 8 g/L Cytodex-3 (GE Healthcare, Sweden) microcarriers. After cell number mounted to about 5×10^6/mL, allantoic fluid containing A/New Caledonia/99/55 was added at 0.01$TCID_{50}$/cell. Incubation was carried out at 34 °C for 3–4 days using porcine trypsin at a concentration of 5 μg/mL [33]. After 96 h cultivation, virus culture broth was harvested and inactivated by 1/5000 (v/v) formalin at 4 °C for 7 days.

2.2. Purification process

Inactivated virus culture broth was clarified by a combination of continuous flow centrifugation at 10,000 × *g* using Beckman Avanti J-25 high performance centrifuge (Beckman, USA) with JCF-Z continuous rotor and prefiltering with Sephadex G-50. To avoid the viral losses due to the retentate in the centrifuge chamber, 1000 mL phosphate buffer (PB, 10 mM, pH 7.2) was added to rinse rotor chamber at the end of the centrifugation for washing out the retentated virus culture. The partially clarified virus culture and the "centrifuge wash" were pooled and used for sequential chromatography processes. An ÄKTAexplorer 100 chromatography system (GE Healthcare, Sweden) was used for chromatography operation. Partially clarified virus batch were loaded multiple times on 20 mL Sephadex G-50 in 10 cm × 2 cm column equilibrated with 10 mM PB at a flow rate of 1 mL/min. G-50 flowthrough was passed a 5 mL HiTrap DEAE FF at a flow rate of 1 mL/min pre-equilibrated with 10 mM PB (pH 7.2) in order to remove Vero cell DNA. The hcDNA adsorbed to DEAE column was eluted with 2 N NaCl. DEAE flowthrough was loaded on column (10 cm × 1.6 cm) packed with 50 mL Cellufine Sulfate (Chisso, Japan) pre-equilibrated with 10 mM PB (pH 7.2) with the flow rate of 1 mL/min. Influenza virions absorbed to Cellufine Sulfate matrix were eluted with 1.5 N NaCl. The CSC eluates were loaded multiple times with 0.1 cv on gel filtration XK 100 columns (100 cm × 1.6 cm) packed with Sepharose 6FF and fractionated by ÄKTAexplorer 100 chromatography system at a flow rate of 5 mL/min. Sepharose 6FF column was equilibrated and eluted with PB (20 mM, pH 7.3) containing 0.65 M NaCl. In order to maintain the stability of viral proteins, all the above processes were operated under 4 °C. Different fractions were collected either in smaller aliquots or as a whole from the column and analyzed for HA activities, protein content, host DNA and protein concentrations or conductivity (Mettler, Sweden). A process flow sheet for upstream and downstream processing is shown in Fig. 1.

2.3. Hemagglutination assay

Hemagglutinin was quantified as described [2]. Serial double dilutions of the samples (100 μL) were performed in round-bottomed 96-well microplates containing 100 μL PBS. Each sample was assayed in duplicates. A chicken red blood cell (RBC) solution (~2.4×10^7 RBCs/mL) was added 100 μL/well and incubated for 90 min at room temperature. The last dilution showing complete