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Downstream processing of MDCK cell-derived equine influenza virus

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Abstract

A microcarrier-based process was used to produce equine influenza virus (A/Equi 2 (H3N8), Newmarket 1/93) in Madin Darby Canine kidney (MDCK) cells. The virus was purified in a sequence of downstream processing steps comprising of depth filtration, inactivation, ultrafiltration (UF) and gel filtration. In the ultrafiltration step, the hemagglutinin (HA) was recovered to 100%. A high increase of neuraminidase (NA) activity indicated the removal of some inhibitory compounds during this step. At the same time, the level of contaminating proteins and DNA was reduced by more than 88%. In the subsequent size exclusion chromatography (Sepharose CL 2B), the recovery of HA and NA in the "virus peak" was 37.8 and 59.8%, respectively compared to the concentrated feed material. Inconsistencies in the overall mass balance for HA and NA (70.0 and 69.2%) during gel filtration indicated non-specific interactions of the inactivated virus to the gel matrix which is supported by a HA recovery of about 50% in shake flask experiments performed as a control. Overall 35.8% of HA and 291.6% of NA were recovered. More than 95.7% of the host cell proteins and 98.7% of the host cell DNA were removed during downstream processing.

Keywords: Chromatography; Downstream processing; Influenza; Virus; MDCK cell and vaccine

1. Introduction

Vaccines play an important role in the prevention, control and eradication of infectious and contagious diseases. Vaccination is the principal means of prophylaxis for human and veterinary use and there is no therapy in view after manifestation of the disease except for passive immunization and few chemotherapeutic successes e.g. against influenza or herpes virus. Though influenza vaccines are still produced in eggs, the cultivation of cells, which are grown in suspension or monolayer culture and are finally infected with virus, is the most important production system today. With increasing safety demands by the Food and Drug Administration (FDA) and the European Agency for Evaluation of Medicinal Products (EMEA) to reduce the levels of possible side effects such as allergic and autoimmune reactions, continuous efforts to improve downstream processing methods are required.

Influenza virus is a lipid-enveloped RNA virus that belongs to the *Orthomyxoviridae* family and causes respiratory infections that result in severe human and animal suffering and high economic losses. For decades, vaccine supply relied on embryonated chicken eggs as a substrate for influenza propagation [1]. However, to cope with a potential shortage of eggs in a pandemic situation [2], to increase the flexibility of production campaigns and to avoid problems related to egg-derived vaccines, i.e. the risk of allergies against egg albumin and the selection of egg adapted virus subtypes, large-scale mammalian cell culture systems were developed for human and veterinary influenza vaccines [3–11]. So far, most publications have focused on upstream

Abbreviations: BEI, binary ethyleneimine; DNA, deoxyribonucleic acid; EDTA, ethylene diamine tetra-acetic acid; FCS, foetal calf serum; GF, gel filtration; KDa, kilodalton; HA, hemagglutinin; MDCK, Madin Darby Canine kidney; MALS, multiangle laser light scattering; MW, molecular weight; $NaHCO_3$, sodium bicarbonate; NA, neuraminidase; PBS, phosphate buffer saline; SDS–PAGE, sodium dodecylsulphate–polyacrylamide gel electrophoresis; UF, ultrafiltration

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processing and virus yields from bioreactors as well as efficacy and safety of final vaccines while comparatively little is reported on downstream processing of cell culture derived viruses [3,6,10,12].

Downstream processing of influenza virus from allantoic fluids (egg-derived influenza virus) usually consists of clarification by centrifugation followed by concentration by ultrafiltration and purification by ultracentrifugation [13]. Earlier, Polson et al. [14] and Polson [15] demonstrated purification by polyethylene glycol precipitation. In addition, several authors describe the use of continuous zonal centrifugation using sucrose [6], potassium tartrate [10] and caesium chloride [16] to purify viruses from cultivation broths. However, while results for final virus yields and host cell DNA per dose load are given, there are no reports on HA recovery or reduction of contaminating DNA and host cell proteins for individual processing steps.

Downstream processing of cell culture derived influenza viruses also necessitates a multi step approach to fulfil pharmaceutical requirements. While it is possible to adapt inactivation and solubilisation procedures originally developed for egg-derived vaccines [4] the differences in starting materials, i.e. allontoic fluids versus cell culture media containing microcarriers and cell debris, usually require additional methods for the efficient purification of viral antigens. One option is the clarification of the cultivation broth by depth filters or separators followed by the concentration of the antigen by crossflow filtration and inactivation [7]. In a next step, virus is purified by one or more chromatography methods, e.g. a combination of size exclusion or anion exchange chromatography, to fulfill (BE versus AE) all pharmaceutical requirements concerning purity, efficacy and safety. Until now, no results have been published that critically evaluate such a process. In the present paper, we report experimental data on the recovery of HA and NA activity of inactivated influenza virus harvests as well as the removal of DNA and contaminating proteins. Typical results obtained for the downstream processing of MDCK cell-derived equine influenza virus (A/Equi 2 (H3N8), Newmarket 1/93) from large-scale microcarrier culture are discussed.

2. Materials and methods

2.1. Preculture in roller bottle and large-scale microcarrier culture

Madin Darby Canine kidney (MDCK) cells (no. 841211903, ECACC, UK) were grown on cytodex 1 microcarriers (Amersham Bioscience, Freiburg, Germany) at 37 °C in a 5 L fermenter (B. Braun Biotech., Melsungen, Germany) containing cell growth medium based on GMEM (Invitrogen/Gibco, Karlsruhe, Germany) supplemented with glucose (final concentration 5.5 g/L; Sigma-Aldrich, Germany), 10% (v/v) fetal calf serum (Invitrogen/Gibco, Karlsruhe, Germany), 2.0 g/L peptone (autoclaved 20%, v/v; International Diagnostic Group, Lancashire, UK) and 4.0 g/L $NaHCO_3$ (Sigma-Aldrich, Germany). Cells were infected at 37 °C with equine influenza virus (A/Equi 2 (H3N8) Newmarket 1/93, NIBSC, UK) in cell growth medium without serum containing low levels (5 mL, 10 mg/mL) of porcine trypsin (Invitrogen/Gibco, Cat No. 27250-018, Karlsruhe, Germany) and 4.5 g/L of glucose. This medium is called virus maintenance medium. MDCK cells were grown in roller bottles (Greiner, Esslingen, Germany, 850 cm^2) containing 250 mL cell growth medium (Sigma-Aldrich, Germany) for inoculum preparation. The detailed procedure is described elsewhere [17].

Virus culture broth was harvested after passing through two depth filters (Polyfil II, 5 μm and 1 μm, with 0.22 and 0.3 m^2 filteration area, respectively; Vokes Filtration Technology, Germany) under positive pressure and inactivated before downstream processing using a final concentration of 1.5 mM binary ethyleneimine (BEI) [18].

2.2. Downstream processing

The partially clarified virus culture was concentrated on a plate type flat sheet 100 kDa MW cut-off ultrafiltration (UF) polyethersulfone membrane (Sartocon 3021466907E-SG, Sartorius, Göttingen, Germany) through a Watson Marlow 505S peristaltic pump equiped with 6.4 mm silicon tubing and the concentrated virus material (UF retentate) was harvested. To collect virus particles adsorbed onto the membranes 500 mL PBS (pH 7.2) was added and circulated without throttling the outlet valve. The inlet and outlet pressures of the UF system were at atmospheric pressure at a flow rate of 18 L/h. The virus washed from the membrane is termed in the following as UF wash. The concentrated virus was fractionated on gel filtration XK 100 columns (100 cm × 1.6 cm; Amersham Bioscience, Freiburg, Germany) packed with Sepharose CL 2B (70–40,000 kDa; Amersham Bioscience, Freiburg, Germany) at a flow rate of 1 mL/min (30 cm/h) at room temperature. The height of the gel bed was 95 cm and the loading volume of the UF retentate 10 and 20 mL, which corresponded to 5 and 10% of the column volume. The pressure of the system increased about 0.3 bar during loading the column whereas the pressure during packing was about 0.2 bar at these operating conditions. Four fractions were collected either in smaller aliquots or as a whole from the column and analysed for HA, NA activities, DNA and protein concentrations (Tables 1 and 2): GF Fraction 1 (the fraction collected before the first peak), GF Fraction 2 (the fraction of the first peak, called virus peak), GF Fraction 3 (the fraction collected after the virus peak), and GF Fraction 4 (the fraction of the second peak containing phenol red and other low molecular weight components of the culture broth). A process flow sheet for upstream and downstream processing is shown in Fig. 1.