Journal of Chromatography A, 1218 (2011) 5279–5285

Contents lists available at ScienceDirect

Journal of Chromatography A

journal homepage: www.elsevier.com/locate/chroma

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

Chunyan He[a,b,*,1], Zhiqiang Yang[a,b,1], Kuitang Tong[a,b]

[a] School of Medicine, Shanghai Jiao Tong University, Shanghai 200025, China
[b] Research Department, Shanghai Institute of Biological Products, Shanghai 200052, China

ARTICLE INFO

Article history:
Received 15 October 2010
Received in revised form 21 February 2011
Accepted 9 June 2011
Available online 17 June 2011

Keywords:
Anion exchange chromatography
Cellufine Sulfate Chromatography
Downstream processing
Human Influenza Virus
Serum-free
Size-exclusion chromatography

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].

* Corresponding author at: School of Medicine, Shanghai Jiao Tong University, 227 Chongqing Road South, Shanghai 200025, China. Tel.: +86 21 63846590 776633; fax: +86 21 63846383.
E-mail addresses: hechyan@hotmail.com (C. He), hello_yzq@163.com (Z. Yang), tongkt@126.com (K. Tong).
[1] Chunyan He and Zhiqiang Yang contributed equally to this work.

0021-9673/$ – see front matter © 2011 Elsevier B.V. All rights reserved.
doi:10.1016/j.chroma.2011.06.043

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

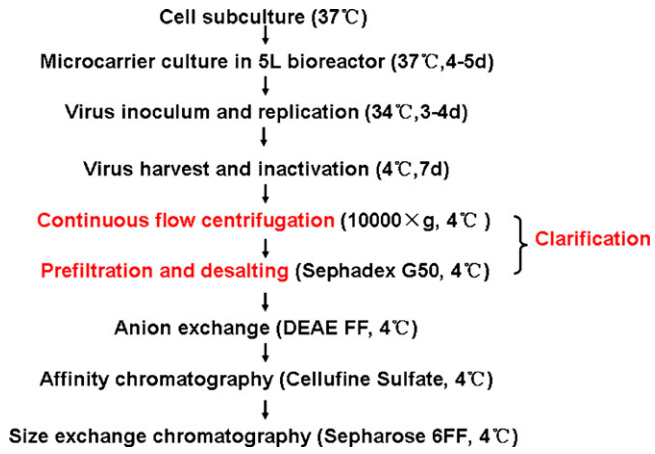

Fig. 1. Upstream and downstream processing of Vero cell-derived human influenza virus A.

hemagglutination was taken as the end point and was expressed as HA titer unit (HAU/0.1 mL).

2.4. Determination of the specific hemagglutinin antigen content

The concentration of hemagglutinin was determined by the single radial immunodiffusion (SRID) [34]. Briefly, 4 mm diameter and 2 mm depth wells were cut in 1% agarose in PBS. The agarose contained 22 μL/mL anti-HA (H1N1, A/New Caledonia/99/55) sheep antisera (NIBSC standard). Samples and A/New Caledonia/99/55 HA positive standards (NIBSC standard) were diluted in 10% Zwittergent (Calbiochem, USA), before application (0.02 mL) to the agarose wells. After 72 h incubation at room temperature, the gels were washed with PBS and stained with 0.3% (v/v) Coomassie blue in bleaching solution (10% acetic acid in 45% methanol). After standard destaining for 24 h, precipitin ring radius for each sample was measured with a micrometer and compared to an influenza A/New Caledonia/99/55) standard (NIBSC) curve.

2.5. Protein assay

Protein concentration was determined by bicinchoninic acid (BCA) protein assay kit (Pierce, USA), calibrated with bovine serum albumin standards according to manufacture instructions. All samples were measured in triplicates.

2.6. Preparation of guinea pig or rabbit IgG against Vero cell proteins

Vero cells grown in serum free medium were adjusted to a concentration of 2×10^7 cells/mL and lysed with three cycles of freezing and thawing. The cell debris was removed by centrifugation at 10,000 × *g* at 4 °C for 10 min. Supernatant was quantitated with Pierce BCA protein assay kit and used for immunization or as standards. Guinea pig or rabbit were immunized with the lysate equivalent to 2×10^7 Vero cells in complete Freund's adjuvant (1:1) (Sigma, USA) and boosted twice at 4 week intervals with the same lysate in incomplete Freund's adjuvant (1:1). One week after the last booster, animals were bled and anti-Vero cell protein IgG was purified with Montage Antibody Purification Kit with PROSEP-A media (Millipore, USA).

2.7. Quantification of host cell-derived proteins

The Vero proteins in samples were measured by ELISA. The anti-Vero guinea pig IgG (3 μg/mL into $NaHCO_3$, pH 9.6) was coated with 100 μL/well into a 96-well microplate (Corning, USA) overnight at room temperature. After three washes with PBST (PBS pH 7.2, 0.05% Tween 20), the plate was blocked with blocking buffer (3% BSA in PBST) for 1 h. Host cell protein standard and samples were serially diluted with blocking buffer and added into wells with 100 μL/well after three washes and incubated for 1 h at 37 °C. The plate was washed for three times and anti-Vero rabbit IgG (1:1000 in blocking buffer) was added with 100 μL/well and incubated for 1 h at 37 °C. After three washes, HRP-conjugated goat anti-rabbit serum was added and incubated for 1 h at 37 °C. After washes with PBST, TMB solution was added with 100 μL/well and incubated for an appropriate time at 37 °C before 1 M H_2SO_4 solution was added to stop the reaction. Color development was measured at 490 nm by a Thermo microplate reader (Thermo, USA).

2.8. Host cell-derived DNA estimation

The content of hcDNA was determined according to Chinese Pharmacopeia [35]. Please see Supplemental Experimental Procedures for detailed information (Supplement 1).

2.9. SDS-PAGE and Western blotting

Samples from culture broth before or after inactivation, clarification, DEAE flowthrough and CSC flowthrough were concentrated 10 fold by Amicon Ultra-15 Centrifugal Filter Unit (Millipore, MW cut-off 3 kDa) followed by two to three washing steps with PBS and used for SDS-PAGE. The dissociation and electrophoresis conditions were as described [6]. SDS-PAGE was carried out with a Mini-Protean II electrophoresis Cell (Bio-Rad, USA) according to the manufacturer's instructions. Protein bands were visualized by Commassie Blue staining. Electrophoretically separated proteins in another two SDS-PAGE gels were transferred to polyvinylidene difluoride membranes and incubated in a blocking buffer (PBST containing 5% BSA) for 1 h. After three washes with PBST anti-HA (H1N1, A/New Caledonia/99/55) sheep antisera (NIBSC) in the 1:1000 ratio or rabbit anti-Vero IgG in 1:500 ratio was added and incubated overnight at 4 °C. After three washes with PBST secondary HRP-conjugated goat anti-sheep IgG or sheep anti-rabbit IgG was added in the 1:2000 ratio and 1 h incubation followed. After three washes TMD substrate was added on the membrane for the colorimetric development of bands.

2.10. Negative stain electron microscopy

Influenza virus in viral fraction from Sepharose 6FF was visualized by negatively transmission electron microscope (JEM 100-CX, Japan) according to the methods described previously [36].

3. Results

3.1. Biosafety tests of cell banks

The Vero cell lines were distributed by ATCC at passage level 120 and fully adapted into serum-free growth after several passages in serum-free medium. The MCB and WCB were established at passage 133 and 137 respectively in serum free medium and have been fully characterized for the presence of a range of specific adventitious viruses (Table 1). The cell banks were tested for sterility, mycoplasma, retroviruses, *in vivo* and *in vitro* adventitious agents and were found to be free of such contamination. Vero cells from the MCB and WCB did not produce palpable tumors during 3 weeks observation period in athymic nude mice. STR profiles of MCB and WCB were CSF1PO:14, D7S820:10, Amelogenin:X, consistent with the STR profiles of Vero cell line in Japanese Collection of Research Bioresources. Master Cell Bank and Working Cell Bank

Table 1
Tests for Vero master cell bank and working cell banks.

Test	MCB	WCB
I Culture and supernatant	Negative	Negative
Bacteria and fungi	Negative	Negative
Mycoplasma	Negative	Negative
II Viral contamination		
1. Direct culture and Hemagglutinin adsorption assay	Negative	Negative
2. *In vitro* assay in different cell lines		
Vero E6	Negative	Negative
MRC-5	Negative	Negative
Vero	Negative	Negative
3. *In vivo* testing (sukling mice, adult mice, guinea pigs, rabbits and embryonated specific pathogen-free eggs)	No evidence of viral contamination	No evidence of viral contamination
4. Reverse transcriptase assay		
(1) Electron Microscope	No evidence of viral contamination	No evidence of viral contamination
(2) Reverse transcriptase assay	Negative	Negative
III Tumorigenesis	No tumor and no histopathologic change	No tumor and no histopathologic change
IV Identification (STR profile)	CSF1PO, 14; D7S820, 10; Am, X.	CSF1PO, 14; D7S820, 10; Am, X.

fulfilled all the requirements of Chinese Pharmacopeia [32] for the manufacture of biological products.

3.2. Purification process

HA activity, total protein, host cell protein and DNA concentrations including a summary of the recovery and the material balances for all process steps of a typical batch are presented in Tables 2 and 3, respectively. A typical virus culture broth harvested from a microcarrier-based cell culture after 4 days cultivation had a DNA content of 100 ng/mL, protein concentration of 182 μg/mL, host cell protein of 115.5 μg/mL, and HA activities of 192 HAU/0.1 mL (Table 2). For biosafety consideration, the virus was inactivated with formalin at 4 °C for 7 days. Egg safety test showed negative for haemagglutination, an indicative of effective inactivation. During inactivation, clarification and subsequent DEAE flowthrough, HA activities fluctuated within ±0.15 log HAU/0.1 mL (+41%/−29% HAU/0.1 mL, linear), which is within the typical error range of such an assay [37]. Inactivation resulted in the reduction of total protein and hcDNA by 6.14 and 2 fold, respectively (Table 3). ELISA results showed the removal of half of Vero cell protein, indicating that host cell protein might precipitate with formalin. Broken microcarriers, cell debris or precipitation caused by inactivation was removed by a combination of continuous flow centrifugation and prefiltration with Sephadex G50. The low salt concentration facilitates the efficient removal of hcDNA in the sequential DEAE unit operation [7]. At the end of continuous flow centrifugation, 1000 mL 10 mM PB (pH 7.4, 2.2 ms/cm) were used to harvest the retentated viral broth in centrifuge chamber and dilute the sample to lower the ionic strength of virus broth (from 28.2 to 18.8 ms/cm). After centrifugation, the loss of HA activity was likely due to the removal of aggregated viral particles caused by inactivation or cell membrane composition containing HA antigen. Sephadex G50 is mainly applied for desalting and buffer exchange. G50 can also be used as a depth filtration matrix. Cell debris was blocked by the gel while influenza virions and soluble impurities flow through. Small molecular salt was retained in G50 resin. After G50, the conductivity in G50 flowthrough was reduced to 6.69 ms/cm. The low ionic strength may facilitate hcDNA binding to AEC resin in the next step. Overall clarification reduced total protein and hcDNA by 1.42 and 1.97 fold, respectively. This suggested that most of hcDNA may be removed with host cell debris. Clarification did not markedly reduce host cell protein, indicating the reduction of total protein might origin from aggregated viral particles or insoluble cell debris. The slightly increase in Vero protein concentration in G50 unit operation was probably caused by methodological accuracy of ELISA.

DEAE unit operation was applied in a negative mode (i.e., virus in the flowthrough). At low salt concentration, hcDNA strongly adsorbed to DEAE matrix. An overall 40 fold reduction in hcDNA was achieved after DEAE FF chromatography. Influenza virions did not adsorb to DEAE matrix and appeared in the flowthrough. HA activity was not markedly affected with fluctuation from 128 to 192 HAU/0.1 mL. The HA activity in DEAE eluate may be caused by cell components containing HA molecules. DEAE chromatography was ineffective in the reduction of total protein (0.88 fold) and host cell protein (1.05 fold). The conductivity of DEAE flow-through was 13.66 ms/cm. The fluctuation of the ionic strength has not been clearly understood.

After DEAE, CSC was used to capture influenza virus in DEAE flowthrough. Influenza virus was adsorbed to CSC column and eluted with 1.5 N NaCl. CSC resulted in concentration factor of HA activity and volume by 134 and 170 fold, respectively. A product yield of 127% based on HA activity was achieved and HA protein in CSC elute was 150 μg/mL (SRID). HA activity was under detection level in CSC flowthrough, indicating that nearly all virions were adsorbed to CSC matrix. CSC also caused the reduction of protein, Vero cell protein and DNA by 2.7, 13.7 and 224 fold, respectively.

The CSC eluate, which was the starting material for Sepharose 6FF, has 32,768 HAU/0.1 mL and 1656.6 μg/mL total protein. The column was loaded multiple times with 0.10 cv of the CSC eluate. The fractionation range of Sepharose 6FF is from 10 kDa to 4000 kDa, hence influenza virions were eluted at the void volume (about 0.5 cv) due to their high molecular weight (Fig. 2). Small solutes were retained in Sepharose 6FF resin. A typical double peak pattern was not obviously observed in the UV trace as the protein content in viral fraction was extremely higher compared to impurities. Due to the high salt concentration in CSC eluate, the conductivity peak at 1 cv implied the appearance of the salt composition (Fig. 2). The UV absorbance of tailing of product fraction was low but not zero, which indicated that partly damaged virions can enter a fraction of pores. The second peak (impurity peak) reported elsewhere [7] was indistinct from the tailing of the first viral faction (0.7–1 cv). Influenza virions in the viral fraction were visualized by scan electric microscope (SEM) (Fig. 3) and viral aggregation was not observed. Some partly broken virions were probably caused by shear damages of previous purification process. In contrast, the impurity fraction only exhibited very low HA activity (<10 HAU/0.1 mL) and protein content (under detection). In Sepharose 6FF, the yield of HA was 63.2%. Based on the initiated

Table 2
HA activities, protein, Vero-derived protein and DNA content in various steps for Vero cell-derived human influenza A downstream processing.

Steps	Volume (mL)	HAU/0.1 mL	Protein (μg/mL)	Vero protein (μg/mL)	HA Protein (μg/mL)	DNA (pg/mL)
Culture broth	3700	192	181.83	117.5	ND	100,000
Inactivation	3700	256	53.33	56.72	ND	50,000
[a]Centrifugation	4700	128	31.58	40.5	ND	ND
G50 flowthrough	4700	128	29.60	41.03	ND	20,000
DEAE flowthrough	4700	192	33.56	39.21	ND	500
DEAE elute	9	2048	695.84	1631.97	ND	500,000
CSC flowthrough	4700	<1	BLD	9.98	ND	400
CSC elute	35	32,768	1656.64	385.37	150	300
SEC virus fraction	120	6044	263.23	8.45	45	100

ND: Not determined.
[a] At the end of continuous flow centrifugation, 1000 mL phosphate buffer (10 mM, pH7.2) was added to wash out the viral retentate in the rotor chamber.

Table 3
Recovery and mass balances during downstream processing of human influenza virus A.

Steps	Concentration factor		% HA recovery	Impurity reduction (fold)		
	Volume	HA		Protein	Host protein	Host DNA
Culture broth	1.00	1.00	100.0	1	1	1
[a]Inactivation	1.00	1.33	133.3	6.14	2.07	2
[a,b]Clarification	0.79	0.75	63.51	1.42	1.09	1.97
[a]DEAE flowthrough	1.00	1.50	150.0	0.88	1.05	40
[a]CSC elute	134.3	170.7	127.1	2.72	13.7	223.8
[a]SEC virus fraction	0.29	0.18	63.2	1.84	13.3	0.88
[c]Overall	30.8	31.5	102.1	21.3	428	30,833

[a] Based on the product fraction in the previous step.
[b] Clarification included high-performance centrifugation and prefiltration with Sephadex G50.
[c] Based on culture broth.

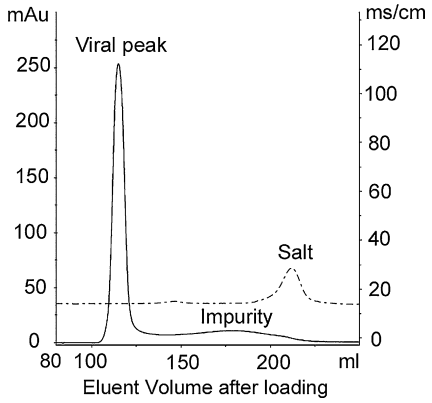

Fig. 2. A typical chromatogram of Sepharose 6FF. Column (100 cm × 1.6 cm) packed with Sepharose 6FF was loaded with 0.1 column volume of Cellufine Sulfate Chromatography eluate. Recording was started after loading. Eluates were traced online for UV absorbance at 280 nm (solid line) and conductivity (diamond line).

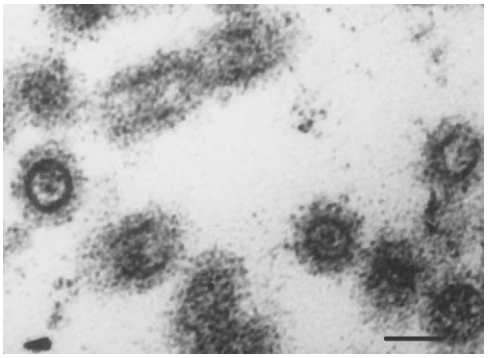

Fig. 3. Negative staining electron microscopy of influenza particles in Sepharose 6FF viral fraction. Bar = 100 nm.

concentration in feed, total protein and Vero cell protein were reduced by 1.84 and 13.3 fold, respectively. SRID assay exhibited that HA content in product fraction was 45 μg/mL. SEC did not efficiently reduce DNA (0.88 fold), indicating co-elution of viral particles and DNA.

Based on the starting virus harvest, the purification process resulted in the recovery of HA activity by 102%, concentration factor of volume and HA activity by about 30 fold and the reduction of total protein, Vero cell protein and DNA by 21.3, 428 and 30,833 fold, respectively. The final product contained 45 μg/mL HA, 263 μg/mL protein, 8.45 μg/mL Vero cell protein and 100 pg/mL DNA.

3.3. Characterization by SDS-PAGE and Western blot

Samples for various purification steps were analyzed by SDS-PAGE (Fig. 4A) and Western blotting (Fig. 4B and C). In order to visualize the effects of purification steps several dilute samples from culture broth before or after inactivation, clarification, DEAE flowthrough and CSC flowthrough were concentrated by 10 fold. The final product mainly contained three bands, 55 kDa, 46 kDa and 27 kDa, which was identified as HA, HA1 and HA2 by Western blotting with specific anti-HA serum. Vero cell protein only appeared in CSC eluate, which was appeared at about 80 kDa. The final product from SEC did not exhibit Vero protein in either SDS-PAGE or Western blotting with anti-Vero IgG, confirmed the removal of most host cell protein.

4. Discussion

Compared to embryonated egg-based technology, cell culture technology for influenza vaccine production offers many advantages including a shorter production time, a more reliable substrate, less risk of contamination and closer matching to the wild influenza strain. Due to the change of the starting materials, the development of downstream processes appropriate for cell culture-derived

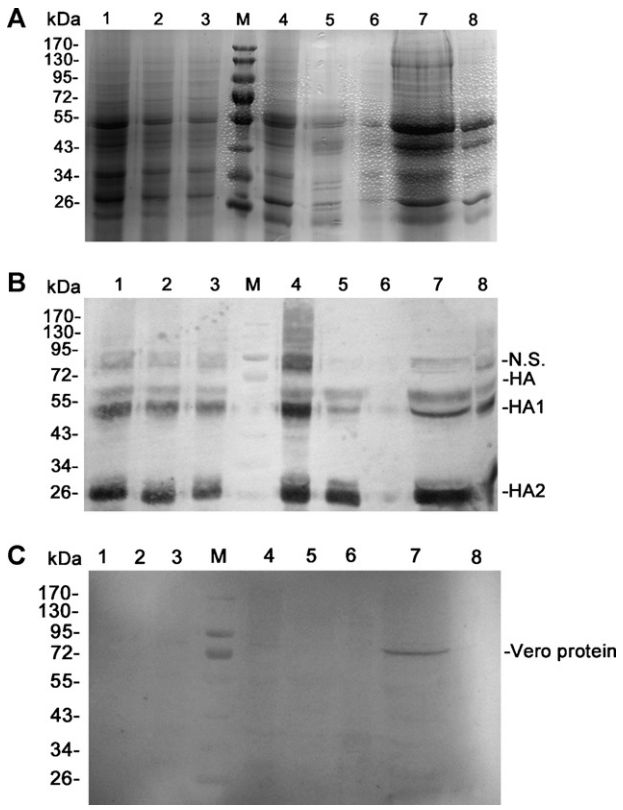

Fig. 4. SDS-PAGE and Western blot of purification process.
(A) SDS-PAGE (B) and (C) Western blotting with anti-influenza virus A serum (B) or by rabbit anti-Vero protein IgG (C). Lane1: culture broth before inactivation (10×); Lane 2: culture broth after inactivation (10×); Lane 3: culture broth after clarification (10×); Lane 4: DEAE flowthrough (10×); Lane 5: DEAE elute (1×); Lane 6: Cerllufine Sulfate flowthrough (10×); Lane 7: Cellufine Sulfate eluate (1×); Lane 8: Sepharose 6FF Virus fraction (1×). N.S. = nonspecific.

influenza vaccine is required. In particular, the residual host cell-derived impurity, which may affect the potency of drugs and cause side effects, has been closely concerned. Under low salt concentration, host genome exhibited stronger binding capacity to Sepharose Q XL resin than influenza virus [15]. We found that weak anion exchange resin DEAE favored the binding of hcDNA versus influenza virus under low ionic strength. Only small amount of influenza virions adsorbed the gel despite low ionic strength (6.69 ms/cm). In comparison, most of hcDNA was adsorbed to DEAE resin. The exact mechanism needs to be investigated. DEAE eluate also exhibited comparable HA activity (2048 HAU/0.1 mL), which indicated partly binding of virions or cell debris containing HA molecules. AEC was reported insufficient to remove hcDNA in MDCK cell-derived influenza virus [7]. The high salting concentration (0.65 M NaCl) used probably accounted for the ineffective separation of hcDNA from virus particles. After AEC, hcDNA was further removed by CSC unit operation. DNA did not specifically bind to Cellufine Sulfate matrix and passed through the column unbound. After CSC chromatography residual DNA was reduced by 224 fold. Contaminating hcDNA often associated with virions and has identical size as virions [7]. Hence, Sepharose 6FF did not effectively remove hcDNA (0.88 fold). Controlling viral aggregation by nonionic detergents or changes of salt concentration may improve the clearance of hcDNA [38]. Influenza virus concentration can be conducted by centrifugation, dia-, ultra-, and microfiltration [15]. Nevertheless, influenza virus particles are fragile to shear force caused by centrifugation or filtration, which often result in damages of virions [39]. It requires laborious efforts for careful selection of membrane pore size and the optimization of operation conditions are needed [39,40]. Generally, a concentration factor of 20–30 can be achieved by these methods. Sartobind anion exchange membrane adsorbers were recently reported to capture influenza virus by 6–7 fold. The shortage of this method was the complete recovery of hcDNA. Lectin-affinity chromatography or membrane [9,25] is also employed to capture influenza virus but the potential toxicity limited their application. CSC has been applied for the capture of MDCK cell-derived influenza viruses [27] and recombinant HA [41]. The overall yields in CSC unit operation vary tremendously in different studies, from 21% [42] to 67.6% [25]. A successful application of CSC depends on the salt concentration. The low salt concentration of DEAE flowthrough was proven suitable for the adsorption of influenza virions. The loss of HA activity was not observed after CSC with the HA yield of 127%. Furthermore, the volumetric concentration factor of 134 and the HA activity enrichment of 170 were achieved, which is difficult to obtain for conventional centrifugation/ultrafiltration methods. As previous reports [7,25], viral aggregation and precipitation were observed and aggravated over time (data not show). The major drawback of CSC is the low productivity due to low density of ligands. Recently, a sulfated cellulose membrane (SCM) chromatography unit operation has been reported to greatly improve the productivity compared to column-based Cellufine Sulfate [43]. Nevertheless, CSC exhibited higher efficiency (more than 200 fold) to remove hcDNA than SCM (50%).

Due to extremely high concentration of influenza virions in CSC eluate, a single viral peak was observed in UV trace instead of a double peak pattern as previous studies [7]. Based on HA activity, the yield of 63% was obtained in Sepharose 6FF. The loss of HA activity may be caused by the long tailing, indicating that some damaged virions entered the porous beads and eluted with cell proteins.

Based on the starting cell culture harvesting, the overall yield of HA activity was 102%. Although HA activity represents the most robust assay in evaluating the purification process of influenza vaccines, the broad accuracy of HA activity (+41%/−29% HAU/0.1 mL, linear scale) and discontinuous assay make it difficult to obtain a closed material balance in many studies [25,40,43,44]. Recently, efforts have been made to optimize the assay of HA activity [37]. By the introduction of a regression procedure, the accuracy of HA activity reduced to +29%/−22% (95% confidence intervals reports). It is possible to obtain a closed mass balance using this optimized HA assay.

According to Chinese Pharmacopeia [45], one injectable dose of a human whole virion inactivated vaccine should contain 15 μg of HA antigen with the total protein of less than 100 μg per strain per dose. For purity requirement, total protein should not exceed six times of the HA content. If Vero cell is used as host cells, the residual DNA content per human dose should not exceed 100 pg [46]. Here, the protein content in the final product was 87 μg protein/15 μg HA and 5.8 fold of HA protein content. The residual amount of hcDNA was 33 pg per 15 μg HA antigen. The final product thereby fulfills the purity requirement for vaccine derived from continuous cell lines. Taken together, the purification process presented was suitable for the production of Vero cell-derived (microcarrier-based culture) influenza vaccine. Viral and host cell protein were not distinguishable by the total protein assay. Hence, the effective removal of host cell protein was not well studied in downstream process of cell-derived influenza vaccine. In this study, an ELISA assay was developed to monitor the change of Vero cell proteins in different unit operations. Vero cell proteins were mainly removed by CSC (13.66 fold) and Sepharose 6FF chromatography (13.3 fold). Thus, the rest proteins in the final product may mainly origin from influenza virus. The slight inconsistency between total protein content and Vero cell proteins in purification process may be owing to the working principles and sensitivity disparity between BCA assay (20–2000 μg/mL) and ELISA (1–100 μg/mL).

5. Conclusion

This study presented a complete downstream process for inactivated cell culture-derived influenza vaccine. This downstream process resulted in high HA recovery and substantial reduction of the impurities. The final product meets the impurity limit required by regulatory authorities. All operation is easy to scale up and is a potential candidate downstream process for cell culture-derived influenza vaccine production.

Competing interest

The authors declare that they have no competing financial interests.

Acknowledgements

The authors like to thank Prof. Chen, Zhihui from Shanghai Institute of Biological Products for his technical assistance. Mr. Wu, Shujun and Ms. Tang Lili from Shanghai Institute of Biological Products, who performed most of cell culture and animal experiments, were acknowledged.

Grant support: This work is supported in part by grants from Shanghai Key Scientific and Technological Project, Shanghai Commission of Science and Technology (044319206), and Rising Star Program of Science and Technology Commission of Shanghai Municipality (Program No. 05QMB1414).

Contributions: Chunyan He (performing the major experiments and writing manuscript), Zhiqiang Yang (responsible for purification), Kuitang Tong (analysis of data and manuscript writing).

Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.chroma.2011.06.043.

References

[1] WHO, Bull. World Health Organ. 73 (1995) 431.
[2] O. Kistner, P.N. Barrett, W. Mundt, M. Reiter, S. Schober-Bendixen, F. Dorner, Vaccine 16 (1998) 960.
[3] H.J. Ehrlich, M. Muller, H.M. Oh, P.A. Tambyah, C. Joukhadar, E. Montomoli, D. Fisher, G. Berezuk, S. Fritsch, A. Low-Baselli, N. Vartian, R. Bobrovsky, B.G. Pavlova, E.M. Pollabauer, O. Kistner, P.N. Barrett, N. Engl. J. Med. 358 (2008) 2573.
[4] C.B. Reimer, R.S. Baker, T.E. Newlin, M.L. Havens, Science 152 (1966) 1379.
[5] N. Bardiya, J.H. Bae, Appl. Microbiol. Biotechnol. 67 (2005) 299.
[6] D.P. Nayak, S. Lehmann, U. Reichl, J. Chromatogr. B Analyt. Technol. Biomed. Life Sci. 823 (2005) 75.
[7] B. Kalbfuss, M. Wolff, R. Morenweiser, U. Reichl, Biotechnol. Bioeng. 96 (2007) 932.
[8] R. Brands, J. Visser, J. Medema, A.M. Palache, G.J. van Scharrenburg, Dev. Biol. Stand. 98 (1999) 93.
[9] L. Opitz, J. Salaklang, H. Buttner, U. Reichl, M.W. Wolff, Vaccine 25 (2007) 939.
[10] L. Gerentes, N. Kessler, G. Thomas, M. Aymard, J. Virol. Methods 58 (1996) 155.
[11] J. Briggs, P.R. Panfili, Anal. Chem. 63 (1991) 850.
[12] M.N. Eglon, A.M. Duffy, T. O'Brien, P.M. Strappe, J. Gene Med. 11 (2009) 978.
[13] A.K. Tina Krober, Katrin Eisold, Bernd kalbfuss, Zimmermann, Udo Reichl, Chem. Eng. Technol. 33 (2010) 941.
[14] A.R. Goerke, B.C. To, A.L. Lee, S.L. Sagar, J.O. Konz, Biotechnol. Bioeng. 91 (2005) 12.
[15] U.R. Michael, W. wolff, Chem. Eng. Technol. 31 (2008) 846.
[16] M.W. Wolff, C. Siewert, S.P. Hansen, R. Faber, U. Reichl, Biotechnol. Bioeng. 107 (2010) 312.
[17] M.D. Butler, B. Kluck, T. Bentley, J. Chromatogr. A 1216 (2009) 6938.
[18] B.D. Kelley, S.A. Tobler, P. Brown, J.L. Coffman, R. Godavarti, T. Iskra, M. Switzer, S. Vunnum, Biotechnol. Bioeng. 101 (2008) 553.
[19] A.A. Shukla, B. Hubbard, T. Tressel, S. Guhan, D. Low, J. Chromatogr. B Analyt. Technol. Biomed. Life Sci. 848 (2007) 28.
[20] A.M. Davidoff, C.Y. Ng, S. Sleep, J. Gray, S. Azam, Y. Zhao, J.H. McIntosh, M. Karimipoor, A.C. Nathwani, J. Virol. Methods 121 (2004) 209.
[21] G. Qu, J. Bahr-Davidson, J. Prado, A. Tai, F. Cataniag, J. McDonnell, J. Zhou, B. Hauck, J. Luna, J.M. Sommer, P. Smith, S. Zhou, P. Colosi, K.A. High, G.F. Pierce, J.F. Wright, J. Virol. Methods 140 (2007) 183.
[22] A.A. Prem Kumar, K.R. Mani, C. Palaniappan, L.N. Bhau, K. Swaminathan, Microbes Infect. 7 (2005) 1110.
[23] N.M. Frazatti-Gallina, R.M. Mourao-Fuches, R.L. Paoli, M.L. Silva, C. Miyaki, E.J. Valentini, I. Raw, H.G. Higashi, Vaccine 23 (2004) 511.
[24] C. Sweet, J. Stephen, H. Smith, Immunochemistry 11 (1974) 295.
[25] L. Opitz, S. Lehmann, A. Zimmermann, U. Reichl, M.W. Wolff, J. Biotechnol. 131 (2007) 309.
[26] I. Kalashnikova, N. Ivanova, T. Tennikova, Anal. Chem. 80 (2008) 2188.
[27] A.M. Palache, R. Brands, G.J. van Scharrenburg, J. Infect. Dis. 176 (Suppl. 1) (1997) S20.
[28] K. Sugawara, K. Nishiyama, Y. Ishikawa, M. Abe, K. Sonoda, K. Komatsu, Y. Horikawa, K. Takeda, T. Honda, S. Kuzuhara, Y. Kino, H. Mizokami, K. Mizuno, T. Oka, K. Honda, Biologicals 30 (2002) 303.
[29] R.S. O'Keeffe, M.D. Johnston, N.K. Slater, Biotechnol. Bioeng. 62 (1999) 537.
[30] E.N. Sorensen, G. Weisman, G.A. Vidaver, Anal. Biochem. 82 (1977) 376.
[31] B. Kalbfuss, D. Flockerzi, A. Seidel-Morgenstern, U. Reichl, J. Chromatogr. B Analyt. Technol. Biomed. Life Sci. 873 (2008) 102.
[32] Chinese Pharmacopoeia 3 (2005) 17.
[33] N.V. Kaverin, R.G. Webster, J. Virol. 69 (1995) 2700.
[34] J.M. Wood, G.C. Schild, R.W. Newman, V. Seagroatt, J. Biol. Stand. 5 (1977) 237.
[35] Chinese Pharmacopoeia 3 (2005) A71.
[36] P. Pushko, T.M. Tumpey, N. Van Hoeven, J.A. Belser, R. Robinson, M. Nathan, G. Smith, D.C. Wright, R.A. Bright, Vaccine 25 (2007) 4283.
[37] B. Kalbfuss, A. Knochlein, T. Krober, U. Reichl, Biologicals 36 (2008) 145.
[38] J.O. Konz, A.L. Lee, J.A. Lewis, S.L. Sagar, Biotechnol. Prog. 21 (2005) 466.
[39] S.R. Wickramasinghe, B. Kalbfuss, A. Zimmermann, V. Thom, U. Reichl, Biotechnol. Bioeng. 92 (2005) 199.
[40] B. Kalbfuss, Y. Genzel, M. Wolff, A. Zimmermann, R. Morenweiser, U. Reichl, Biotechnol. Bioeng. 97 (2007) 73.
[41] P. Vanlandschoot, E. Beirnaert, S. Neirynck, X. Saelens, W.M. Jou, W. Fiers, Arch. Virol. 141 (1996) 1715.
[42] A.S.M. Peterka, et al., WO 2008/006780 A1 (2008).
[43] L. Opitz, S. Lehmann, U. Reichl, M.W. Wolff, Biotechnol. Bioeng. 103 (2009) 1144.
[44] M.W. Bernd Kalbfuss, Liane Geisler, Alexander tappe, Ranil Wichramasinghe, Volkmar Thom, Udo Reichl, J. Membr. Sci. 299 (2007) 251.
[45] Chinese Pharmacopoeia 3 (2005) 124.
[46] Chinese Pharmacopoeia 3 (2005) 100.