Journal of Chromatography A, 1290 (2013) 36–45

Contents lists available at SciVerse ScienceDirect

Journal of Chromatography A

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

Purification of infective baculoviruses by monoliths

Petra Gerster[a], Eva-Maria Kopecky[a], Nikolaus Hammerschmidt[a], Miriam Klausberger[a], Florian Krammer[a], Reingard Grabherr[a], Christa Mersich[b], Lidija Urbas[b], Petra Kramberger[b], Tina Paril[b], Matthias Schreiner[c], Katharina Nöbauer[d], Ebrahim Razzazi-Fazeli[d], Alois Jungbauer[a,*]

[a] Department of Biotechnology, University of Natural Resources and Life Sciences Vienna, Vienna, Austria
[b] BIA Separations GmbH, Villach, Austria
[c] Department of Food Science and Technology, University of Natural Resources and Life Sciences Vienna, Vienna, Austria
[d] VetCore Facility for Research, University of Veterinary Medicine Vienna, Vienna, Austria

ARTICLE INFO

Article history:
Received 12 January 2013
Received in revised form 13 March 2013
Accepted 15 March 2013
Available online 25 March 2013

Keywords:
CIM tube
Monoliths
Baculovirus
Ion exchange chromatography
Infectivity
Dynamic binding capacity

ABSTRACT

A chromatographic process based on monoliths for purification of infective baculovirus without prior concentration step has been established. Baculovirus produced in *Spodoptera frugiperda* cells (Sf-9) were harvested by centrifugation, filtered through 0.8 μm filters and directly loaded onto radial 1 mL anion exchange monoliths with a channel size of 1.5–2.0 μm operated at a volumetric flow rate of one bed volume per minute. Optional an epoxy monolith was used as pre-column to reduce interfering compounds and substances influencing the capacity of anion exchange monoliths for baculovirus infectious virus could be eluted with a step gradient at salt concentrations of 440 mM NaCl. Recovery of infectious virus was highly influenced by composition and age of supernatant and ranged from 20 to >99% active baculovirus. Total protein content could be reduced to 1–8% and DNA content to 38–48% in main virus fraction. Infective virus could be 52-fold concentrated within 20.5 h and simultaneously an 82-fold volume reduction was possible when loading 1150 mL (2.1×10^8 pfu/mL) onto 1 mL scale support.

1. Introduction

Baculovirus is widely used in the pharmaceutical industry for the production of recombinant proteins and for human gene therapy applications [1–4]. The high demand for pure baculovirus preparations requires an efficient purification procedure to remove impurities such as host cell protein and host cell DNA. Monoliths are a new generation of chromatography materials which are suited for separation of bionanoparticles such as viruses [5].

The most commonly used lepidopteran cell lines for production of baculovirus are isolated from *Spodoptera frugiperda* (Sf-9 and Sf-21) and *Trichoplusia ni* (Tn-5 or High Five™) [6]. For biotechnological applications *Autographa californica* M nuclear polyhedrosis virus (*Ac*MNPV) is the most commonly used baculovirus [7]. It is an enveloped, rod shaped virus of 40–50 nm in diameter and 200–400 nm in length.

Conventional purification procedures include ultracentrifugation and tangential flow ultrafiltration [8]. Although tangential flow ultrafiltration is very efficient in terms of virus enrichment, irreversible fouling of the membranes has been observed. The yield of active virus after ultracentrifugation is extremely low and has been reported in the range of 1% [9]. Chromatographic purification steps include size-exclusion chromatography (SEC) [10], cation-exchange membranes [11] and affinity chromatography by Concanavalin A [9]. However, SEC requires a very time consuming ultracentrifugation step and overnight virus re-suspension in buffer before loading and dilutes the product. Ion exchange chromatography is an option to recover infectious virus particles. For purification of viruses, conventional chromatography suffers from low dynamic binding capacity, because the pore size is too small to allow diffusion into the beads and the internal surface cannot be accessed.

Membrane adsorbers have been applied for baculovirus purification [11,12] because they offer large pores. The drawback of membrane chromatography is the large axial dispersion which reduces resolution [13]. Monolithic supports are the material of choice for virus purification because they have channels with diameters larger than 1000 nm and offer a large surface area for adsorption [5]. The mass transport within monoliths is by convective flow and is therefore independent of diffusion [5]. The process times can be reduced substantially, which may result in a product of higher activity and less aggregation. For that reason we selected monoliths for purification of baculovirus from cell culture

* Corresponding author at: Muthgasse 18, 1190 Vienna, Austria.
Tel.: +43 1 476546226; fax: +43 1 476546675.
E-mail address: alois.jungbauer@boku.ac.at (A. Jungbauer).

0021-9673/$ – see front matter © 2013 Elsevier B.V. All rights reserved.
http://dx.doi.org/10.1016/j.chroma.2013.03.047

supernatant. Monoliths have also been described for the concentration, purification and detection of other viruses, e.g., tomato mosaic virus [14,15] and rotavirus [16]. Another advantage of using monoliths is the simple conversion to large scale [17–19].

The goal of this work was the purification of baculovirus from insect cell culture supernatant using monoliths. Our intention was to purify infectious virus particles and we sought a method that would capture the virus from the clarified supernatant without further handling. We have established a method for purification and concentration of baculovirus from crude cell culture supernatant clarified by dead end filtration and a chromatographic step using anion exchange monolith.

2. Material and methods

2.1. Cultivation of Sf9-cells

Sf-9 cells (*S. frugiperda*, CRL-1711; ATCC, Rockville, USA) were cultivated in modified IPL-41 medium [20] containing 3% heat-inactivated FCS (Biochrom AG, Berlin, Germany) supplemented with yeastolate and a lipid/sterol cocktail at 27 °C. For virus amplification 1× penicillin/streptomycin (100×; 10.000 IE) (Biochrom AG, Berlin, Germany) was added to cell culture medium. Cells were passaged every 4–5 days, either at a cell density of 2×10^6 cells/mL or at confluences of 90–100%.

2.2. Virus amplification

Sf-9 cells were infected with baculovirus AcMNPV (*A. californica* nuclear polyhedrosis virus, VR-1345; ATCC, Rockville, USA) at a MOI of 3 and cell density of 1×10^6 cells/mL. Infection was performed in Fernbach flasks and cells were incubated for 3 days at 27 °C while stirring. Baculovirus was harvested by two centrifugation steps at $1000 \times g$ and $4000 \times g$ relative centrifuge force for 10 min each (Heraeus Megafuge 16, Thermo Scientific, Rockford, IL USA). The cell culture supernatant containing baculovirus was divided into aliquots and stored at 4 °C.

2.3. Benzonase digest

Clarified culture supernatant was treated for 1 h with Benzonase purity grade II (Merck KgA, Darmstadt Germany) at a final concentration of 150,000 units per liter.

2.4. Anion exchange chromatography

Purification of 0.8 μm filtered (Millex AA filter, Millipore Bedford, MA, USA) baculovirus containing culture supernatant was conducted by 1 mL radial flow monoliths, either CIM (Convective Interaction Media) DEAE or CIM QA and CIM Epoxy was optionally used as a pre-column (BIA Separations, Ajdovščina, Slovenia). HEPES buffer (20 mM) with or without 200 mM NaCl, pH 7.2, was used for equilibration and 20 mM HEPES, 1 M NaCl, pH 7.2, for elution during initial chromatographic experiments. For further optimization and stepwise gradient experiments, the buffer system was changed to loading conditions of 50 mM HEPES, 200 mM NaCl, pH7.2, and elution was performed with 50 mM HEPES, 1 M NaCl pH 7.2. Regeneration of QA was carried out by 2 M NaCl, 1 M NaOH for 60 bed volumes at a flow rate of 1 mL/min. Epoxy was regenerated under the same conditions, followed by 50% isopropanol treatment for 10 bed volumes at a flow rate of 1 mL/min and 10 bed volumes at one half of the flow rate to remove tightly bound hydrophobic material. All runs were conducted on an ÄKTA explorer 100 (GE Healthcare, Uppsala, Sweden) using a flow rate of 1 mL/min. Conductivity, pH and absorbance at 280 and 260 nm for protein and DNA detection was monitored simultaneously during the purification. Elution fractions of 1 mL were collected, pooled according to chromatogram and analyzed for total protein, DNA content and virus infectivity.

Before loading and after regeneration, a pressure flow curve was determined measuring the pressure drop at flow rates from 1 to 20 mL/min. In addition, the binding capacities of bovine serum albumin and phosphate ions were determined.

2.5. Plaque forming assay

Plaque forming assays were performed in Cellstar 6 Well Cell Culture Multiwell Plates (Greiner bio one, Frickenhausen, Germany) with Sf-9 cells. Cells were seeded at a cell density of 1.5×10^5 cells/mL/cm^2 and incubated for at least 1 h. Serial 10-fold dilutions of sample material were prepared in ILP-41 medium as duplicates. Excess medium was removed from cells; diluted samples were added and incubated for 1 h. After the samples for dilutions were removed, the cell layer was covered with 2 mL agarose overlay (1% Agarose (Sigma Aldrich, St. Louis, MO, USA) in IPL-41 medium supplemented with 10% heat inactivated FCS (Biochrom AG, Berlin, Germany) and 1× pen./strep. (Biochrom AG, Berlin, Germany)) and then incubated for 6 days at 27 °C. Plaques were visualized by staining with 200 μL 1 mg/mL MTT in PBS (Sigma Aldrich, St. Louis, MO, USA). Titers were estimated according to method described by Possee and King [21].

2.6. SDS-PAGE and Western blot

Electrophoresis was performed under reducing conditions on NuPAGE bis/tris gels 4–12% (Invitrogen, Carlsbad, CA, USA) in accordance to manufacturer's instructions under SDS-MES running conditions. Protein bands were visualized by EZBlue™ Gel Staining Reagent (Sigma Aldrich), based on Coomassie Brilliant Blue G-250, or silver staining (Merck, Darmstadt, Germany). After SDS-PAGE, proteins were transferred to a nitrocellulose membrane (Whatman, Dassel, Germany). The membrane was blocked with 3% BSA in PBS-T (0.1%, w/v Tween-20 in PBS) for 2 h. Incubation with primary antibody against baculovirus gp64 AcV5 (Santa Cruz biotechnology, California, USA), diluted 1:500 in PBS-T containing 1% BSA for 2 h was followed by secondary antibody incubation with anti mouse-IgG conjugated with alkaline phosphatase (Sigma Aldrich, St. Louis, MO, USA), diluted 1:1000 in PBS-T with 1% BSA for 1 h. Detection was carried out by Lumi Phos™ (Thermo Scientific, Rockford, IL USA) on Lumi Imager (Boehringer Ingelheim, Ingelheim, Germany).

2.7. Determination of DNA- and protein-content

DNA content in samples was determined by Quant-iT™ PicoGreen® dsDNA kit (Invitrogen). For determination of protein concentration, commercially available Coomassie blue G-250-based protein assay dye reagent was used (Bio-Rad Laboratories, Hercules, CA, USA). Both, DNA and protein assays were performed according to the particular manufacturer's instruction in 96-well plate format and signals were measured by Genius Pro plate reader (Tecan, Männedorf, Switzerland).

2.8. Transmission electron microscopy (TEM)

For negative staining, 300-mesh copper grids, coated with Pioloform film, stabilized by carbon evaporation, and freshly glow discharged, were floated for one min, face down, on the surface of approximately 10 μL of a virus suspension in water. Subsequently, the samples were stained on one drop of a 1% uranyl acetate solution in water for one min. After removal of the excess stain by blotting with filter paper, the grids were air-dried