DEVELOPMENT AND CHARACTERIZATION OF AFFINITY AND PSEUDO-AFFINITY BASED METHODS FOR CELL CULTURE-DERIVED INFLUENZA VIRUS CAPTURING

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February 2010 Lars Opitz

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Abbreviations

AB1, AB2, AB3	adsorption buffer 1, 2 and 3
AC	affinity chromatography
AEX	anion-exchange chromatography
AIL	*Artocarpus integrifolia* lectin, Jacalin
CEX	cation-exchange chromatography
CSR	Cellufine® sulphate resin
DNA	deoxyribonucleic acid
dsDNA	double stranded deoxyribonucleic acid
EB1, EB2	elution buffer 1 and 2
EEL	*Euonymus europaeus* lectin
ECL	*Erythrina christagalli* lectin
FCS	fetal calf serum
FPLC	fast protein liquid chromatography
GMEM	Glasgow minimum essential medium
HA	hemagglutinin
HIC	hydrophobic interaction chromatography
IEX	ion-exchange chromatography
IMAC	immobilized metal affinity chromatography
IP	isoelectric point
LAC	lectin affinity chromatography
M1	matrix protein 1
M2	matrix protein 2 (ion channel)
MAL I	*Maackia amurensis* lectin I
MDBK	Madin Darby bovine kidney
MDCK	Madin Darby canine kidney
MF	microfiltration
NA	neuraminidase
NA-activity	neuraminidase activity
PA	component of influenza RNA-polymerase
PA-I	*Pseudomonas aeruginosa* lectin
PAC	pseudo-affinity chromatography
PB1	component of influenza RNA-polymerase

PB2	component of influenza RNA-polymerase
PBS	phosphate buffered saline
PNA	*Arachis hypogaea* agglutinin
PVDF	polyvinylidene difluoride
RCA	*Ricinus communis* agglutinin
RNA	ribonucleic acid
cRNA	complementary RNA
mRNA	messenger RNA
vRNA	viral RNA
RNP	ribonucleoprotein
SCM	sulphated cellulose membranes
SEC	size-exclusion chromatography
SNA	*Sambucus nigra* bark lectin
SOP	standard operating procedure
SPR	surface plasmon resonance
SRID	single radial immunodiffusion assay
UF	ultrafiltration
WHO	World Health Organization

Symbols

A	Absorption [-]
C	concentration of free target P [M]
c_{DNA}	DNA concentration [ng/ml] or [µg/ml]
c_{HA}	hemagglutinin concentration [µg/ml]
c_L	ligand concentration in solution [mol l^{-1}]
c_{prot}	protein concentration [µg/ml]
d	dilution factor HA-activity assay [-]
HA-activity	hemagglutination activity [HAU/ml] or [kHAU/ml]
i_{prot}	protein impurity [µg/µg HA] or [µg/kHAU]
i_{DNA}	DNA impurity [ng/µg HA] or [µg/kHAU]
K_a	association constant [M^{-1}]
K_d	dissociation constant [M]
k_a	association rate constant [$M^{-1}s^{-1}$]
k_d	dissociation rate constant [s^{-1}]
R	response signal [RU]
R_{max}	maximum response signal [RU]
t	time [s]
ε	molar absorption coefficient [l $mol^{-1}m^{-1}$]

Abstract

Influenza is one of the most worldwide-spread diseases, which infects several million people every year. Besides antiviral medical treatments, prophylactic vaccinations are crucial for controlling seasonal influenza epidemics. Hence, every year large amounts of vaccine doses have to be produced. Conventionally, embryonated chicken eggs are used for human influenza vaccine production. However, this production process has only a limited scalability. In addition, these vaccines contain egg-derived proteins, which may cause allergic reactions. Hence, cell culture-based vaccine production processes have been developed, which require an adapted downstream processing strategy for virus purification.

The scope of this dissertation was the development of affinity- as well as pseudo-affinity-based chromatographic unit operations for the downstream processing of cell culture-derived influenza virus particles. Therefore, two major approaches were investigated: lectin-based affinity chromatography and sulphated cellulose matrices-based pseudo-affinity chromatography. In both fields membrane- and bead-based techniques were considered and compared.

Lectin affinity chromatography was developed first for the Madin Darby canine kidney (MDCK) cell culture-derived human influenza virus A/Puerto Rico/8/34 (H1N1). This dissertation showed that the α1,3-galactose-specific *Euonymus europaeus* lectin (EEL), immobilized on polymer beads, is a suitable ligand for affinity purification of glycosylated viral envelope proteins such as the hemagglutinin. The dissociation of the virus-ligand complex was done by competitive elution with lactose. More than 90% of the influenza virus hemagglutination activity was recovered in the product fraction, while the majority of host cell proteins and nucleic acids were depleted.

For chromatography, matrix selection plays an important role regarding purification efficiency. Therefore, different matrices for EEL as ligand were screened. These supports included stabilized reinforced cellulose membranes, polymer and porous glass particles, cellulose and agarose beads. Strong virus binding was achieved by EEL-modified cellulose membranes and polymer beads. Furthermore, reinforced cellulose membranes had a far better binding capacity than other tested adsorbents.

To determine the general applicability of EEL-affinity chromatography, studies were extended for two other MDCK cell-derived influenza virus strains

(A/Wisconsin/67/2005 (H3N2), B/Malaysia/2506/2004). Both virus strains were captured efficiently by the ligand EEL. These results emphasized the EEL-affinity chromatography as a valuable technique for capturing MDCK cell-derived influenza virus particles. Additionally, the impact of host cells on lectin affinity chromatography has been evaluated. In contrast to findings with viruses propagated in MDCK cells, Vero cell-derived influenza virus A/Puerto Rico/8/34 bound to β1,4-galactose-specific *Erythrina christagalli* lectin (ECL) but only very limited to the α1,3-galactose-specific EEL.

The second major project part of this dissertation describes a capturing method for influenza viruses (A/Wisconsin/67/2005 (H3N2), A/Puerto Rico/8/34 (H1N1), B/Malaysia/2506/2004) using sulphated reinforced cellulose membranes. Purification efficiency with regards to viral yield as well as total protein and host cell dsDNA depletion was directly compared to commercially available cation-exchange adsorbers and to column-based Cellufine® sulphate resin. With the sulphated membranes, high product recoveries and contaminant reductions were possible. Due to a fast binding kinetic and a low back pressure, these membrane adsorbers enabled the capturing process to be operated at an increased flow rate leading to significantly enhanced productivity. Hence, sulphated membrane adsorbers are a valuable choice for industrial influenza vaccine purification processes.

Zusammenfassung

Influenza ist eine weltweit verbreitete Infektionskrankheit, an der jährlich mehrere Millionen Menschen erkranken. Um saisonale Epidemien zu kontrollieren, spielen vorbeugende Impfungen neben antiviraler medikamentöser Behandlung eine sehr wichtige Rolle. Aus diesem Grund werden jährlich große Mengen an Influenzaimpfstoffen produziert. Die traditionelle Herstellung humaner Influenza-Impfstoffe erfolgt in Hühnereiern. Diese Methode besitzt nur eine begrenzte Skalierbarkeit. Außerdem können solche Impfstoffe allergische Reaktionen auf Hühnereiweiße hervorrufen. Alternativ dazu wurde in den letzten Jahren die Virusproduktion in Säugetierzellkulturen etabliert. Darauf aufbauend sind neue und innovative Aufreinigungsstrategien für Influenzaviren notwendig.
Das Ziel dieser Dissertation war die Entwicklung von affinitäts- und pseudo-affinitätschromatografischen Aufreinigungsmethoden für Influenzaviren aus Zellkulturüberständen. Dafür wurden zwei Methoden näher untersucht: die Lektin-Affinitätschromatographie und die auf sulfatierten Zellulosematrizen basierende Pseudo-Affinitätschromatographie. In beiden Fällen verglich man Membran-Adsorption und gelbasierende Säulenchromatographie miteinander.

Zunächst wurde die Lektin-Affinitätschromatographie für den in Hundenierenzellen (Madin Darby canine kidney, MDCK) produzierten Influenzavirusstamm A/Puerto Rico/8/34 (H1N1) etabliert. Es wurde gezeigt, dass das α1,3-Galaktose spezifische *Euonymus europaeus* Lektin (EEL), immobilisiert auf Polymerpartikel, ein geeigneter Ligand für glykosylierte Influenzavirushüllproteine (z.B. Hämagglutinin) ist. Die Viren wurden desorbiert durch kompetitive Verdrängung mit Laktose. Dabei betrug die Wiederfindung der Influenzavirus-Hämagglutinationsaktivität in der Produktfraktion mehr als 90% während Großteile der Wirtszellproteine und –nukleinsäuren abgetrennt worden.
Da die Chromatographiematrix ein wichtiger Parameter ist, wurde die Verwendbarkeit des Liganden EEL an verschiedenen Matrizen, wie z.B. stabilisierte Zellulosemembranen sowie poröse Polymer-, Glas-, Zellulose- und Agarosegelpartikel, untersucht. Hohe Virusadsorptionen erreichte man durch EEL-modifizierte Zellulosemembranen und Polymerpartikel, wobei die Membranadsorber

höhere Bindungskapazitäten bezogen auf die immobilisierte Ligandenmenge besaßen.
Die Übertragbarkeit der Methode wurde an zwei weiteren in MDCK-Zellen produzierten Influenzavirusstämmen (A/Wisconsin/67/2005 (H3N2), B/Malaysia/2506/2004) gezeigt. Die Ergebnisse zeichnen die EEL-Affinitätschromatographie als eine effiziente Methode aus, um Influenzaviren aus MDCK-Zellkulturbrühen abzutrennen. Zusätzlich wurden die Auswirkungen unterschiedlicher Wirtszellen während der Virusproduktion auf die Lektin-Affinitätschromatographie untersucht. Influenzaviruspartikel A/Puerto Rico/8/34 aus Vero-Zellkulturen adsorbierten besser an das β1,4-Galaktose spezifische *Erythrina christagalli* Lektin (ECL) als an das α1,3-Galaktose spezifische EEL.

Als eine alternative Methode wurde die Aufreinigung von Influenzaviren (A/Wisconsin/67/2005 (H3N2), A/Puerto Rico/8/34 (H1N1), B/Malaysia/2506/2004), welche auf sulfatierten stabilisierten Zellulosemembranen beruht, beschrieben. Dabei wurden sowohl die Virusausbeute als auch die Abreicherung der Wirtszellproteine und –nukleinsäuren mit Ergebnissen von kommerziell erhältlichen Kationenaustauschermembranen und sulfatierten Zellulosepartikeln (Cellufine® sulfate) verglichen. Mit den sulfatierten Membranadsorbern waren hohe Virusausbeuten und gute Kontaminantenabreicherungen möglich. Aufgrund schneller Bindungskinetik und niedrigem Rückdruck sind diese sulfatierten Membranadsorber bei höherer Fließgeschwindigkeit anwendbar und stellen daher eine attraktive Methode für die industrielle Aufreinigung von Influenzaviren zur Impfstoffproduktion dar.