Masterarbeit

Preparation of the gp120 Subunit of the HIV-1 Envelope-Protein – comparing the human monoclonal Antibodies 2G12 and B12 in Affinity Chromatography

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Kurzfassung

Mit ca. 34 Millionen Infizierten im Jahr 2011 ist die HIV Pandemie noch immer ein großes weltweites Problem. Durch seine Schlüsselrolle bei der Infektion stellt das virale Hüllprotein gp120 ein höchst interessantes Ziel für den Einsatz als Forschungsreagenz oder auch als möglicher Bestandteil eines eventuellen Impfstoffs dar. Die Herstellung reinen Proteins mittels Fermentation genetisch veränderter Zellen und anschließender Aufreinigung ist in der pharmazeutischen Industrie weit verbreitet. Bei der Reinigung des Proteins aus der Fermentationsbrühe sind hoch-spezifische Reinigungsschritte wie sie etwa die Immunoaffinitätschromatographie darstellt von großem Nutzen. Dabei wird die spezifische Bindung von Antikörper und Antigen ausgenutzt. Polymun Scientific hatte 2009 zwei gp120-spezifische Antikörper im Produktsortiment deren Eignung für den Einsatz in dieser Art der Chromatographie untersucht werden sollte. Es konnte gezeigt werden, dass sowohl der Antikörper B12 als auch der Antikörper 2G12 für den gewünschten Einsatzzweck in Frage kommen, wobei 2G12 aufgrund der besseren dynamischen Bindungskapazität auf der präparierten Säule geeigneter erscheint.

Abstract

With approximately 34 million infected individuals in the year 2011, the HIV pandemic keeps being a worldwide problem. Due to its essential role in the infection, the viral envelope protein gp120 is a highly interesting target for use as research reagent or even as component of an eventual vaccine. The preparation of pure protein by fermentation of genetically modified cells and consecutive purification are common processes in the pharmaceutical industry. Highly specific purification steps like immunoaffinity chromatography are of great advantage. This process utilizes the specific binding of an antibody to its antigen. In the year 2009 Polymun Scientific offered two gp120-specific antibodies in the portfolio which were tested on suitability in this type of chromatography. It could be shown that both antibodies, namely B12 and 2G12 can be used as ligands for the chromatography. However, due to the better dynamic binding capacity of the 2G12 column, this antibody should be favoured.

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„Die Lösung ist immer einfach, man muss sie nur finden.“
(Alexander Solschenizyn)

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1. Introduction and Aims

With 1.7 million deaths due to acquired immune deficiency syndrome (AIDS) in 2011, this disease keeps being a worldwide problem. The causative agent of AIDS is the human immunodeficiency virus type 1 (HIV-1). The Joint United Nations Program on HIV/AIDS estimates approximately 34 million individuals infected with HIV worldwide in 2011 and 2.5 million new infections in the same year (UNAIDS, 2013).

Although several antiretroviral therapies (ART) are approved for treating HIV-infection, the most desirable treatment – an effective prophylactic treatment – is not available yet (Jeang, Huang, 2009).

Infection with HIV-1 starts with the viral entering process in which the viral envelope protein gp120 binds to CD4 on the target cell and in combination with the following co-receptor binding triggers the fusion of the viral and cytoplasmatic membranes thus enabling the virion core to enter the cell (Wagner et al., 2008).

Due to this role and the exposure of gp120 on the viral membrane, preparation of pure recombinant gp120 is important for use as a research reagent or even as a component of a potential HIV vaccine (Jeffs et al., 1996; Lichterfeld et al., 2012).

The high specificity of the antibody – antigen interaction which is used in affinity chromatography makes it a favourable method for purification of recombinant gp120 out of a fermentation broth (GE Healthcare, 2007).

When this work was started Polymun Scientific offered two broadly neutralizing antibodies against gp120 in its product portfolio: 2G12 and B12. It was obvious to test suitability of these antibodies for use in affinity chromatography. However, it is often difficult to find proper process conditions that allow a repeatable purification procedure.

This leads to following questions:

- Are Polymun's human monoclonal antibodies 2G12 and B12 suitable for repeatable and reliable purification of recombinant gp120 by immunoaffinity chromatography?
- Are there significant differences between the two antibodies when used in immunoaffinity chromatography?

2. Basic Principles

2.1. The Role of gp120 in the Replication of Human Immunodeficiency Virus Type 1 (HIV-1)

The surface glycoprotein of HIV-1 is 120 kDa overall and is therefore called gp120. It is used by the virus in the first step of the multistep entering process which starts with the high affinity binding of gp120 to CD4. The CD4 binding site of gp120 is well hidden in a small cleft where it is protected from binding and blocking reactions of most antibodies. The binding of gp120 to CD4 results in a conformational change which leads to the exposure of a co-receptor binding site. This co-receptor is usually CCR5 or CXCR4 and its binding enables a second conformational change in gp120 which causes the transmembrane glycoprotein (gp41) to mediate fusion of viral and cytoplasmic membranes which in turn enables the virion core to enter the cytoplasm of the target cell. The entering process is shown in Figure 1 (Wagner et al., 2008).

Gp120 and gp41, both originate from the combined precursor gp160 by endoproteolytic cleavage in the Golgi-complex. After cleavage the proteins remain associated by noncovalent molecular interactions (Stein & Engleman, 1990).

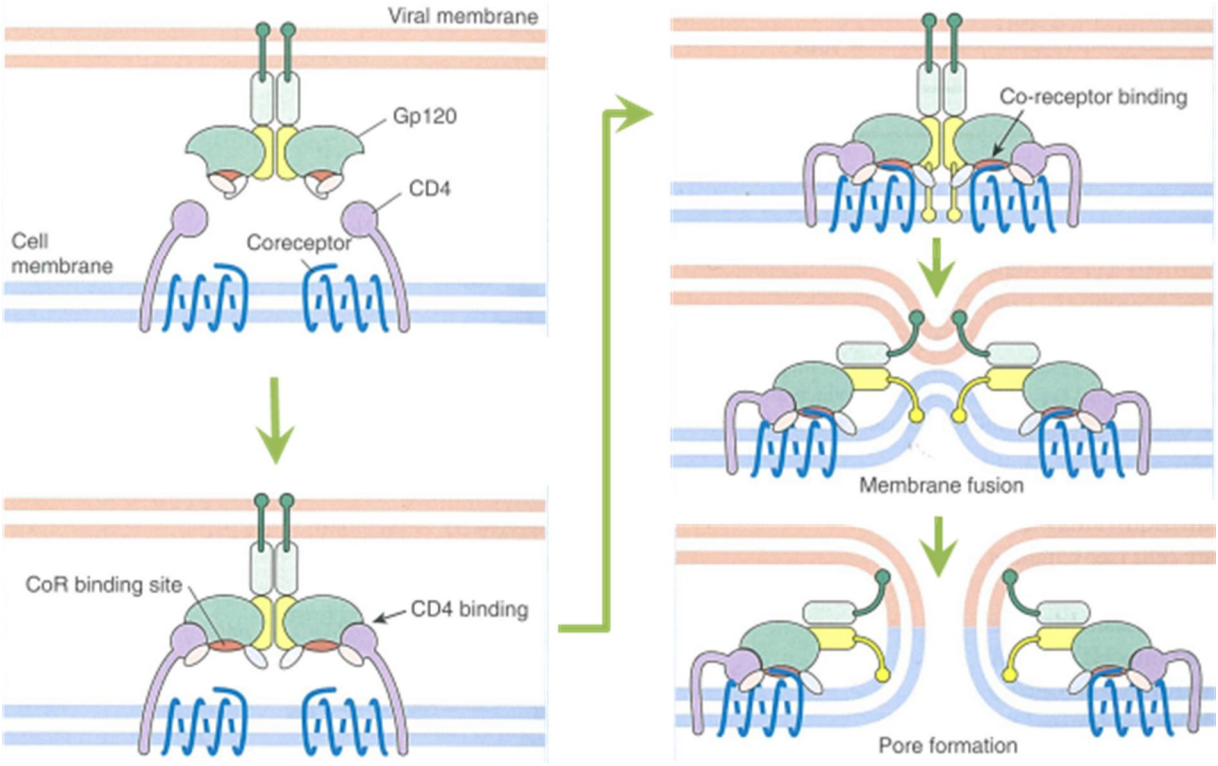


Figure 1: HIV-1 entering process. The binding of gp120 to CD4 enables co-receptor binding resulting in a conformational change which in turn leads to pore formation mediated by gp41 (adapted from Wagner et al., 2008).

2.2. Epitopes for 2G12 and B12 on gp120

The HIV-1 envelope spike comprises of the two noncovalently bound proteins gp120 and gp41 where gp120 represents the surface protein and gp41 forms the transmembrane protein. Three of these heterodimers generate a trimeric spike. As a result of this structure, a number of conserved epitopes present on monomeric gp120 or gp41 are at least partially buried in the interfaces formed due to the trimeric structure of the native spike (Zwick et al., 2003).

However, some broadly neutralizing antibodies against HIV-1 exist which seem to overcome these steric limitations as their epitopes are not hidden in the trimeric envelope spike. The antibodies used in this work both belong to this group of antibodies.

B12 recognises an epitope in a highly conserved region which partially overlaps the CD4-binding site (Saphire et al., 2001).

In contrast, 2G12 recognises an epitope which is formed by high-mannose and/or hybrid glycans on the surface of gp120 close to, but distinct from the co-receptor binding site (Sander et al., 2002).

2.3. Production of recombinant Proteins in Chinese Hamster Ovary Cells

In this work a Chinese hamster ovary cell line transfected with pEE6HCMVgp120GS was used for production of recombinant gp120.
This cell line uses the function of glutamine synthetase (GS) on the expression vector to enable the cells to grow in glutamine free medium as the enzyme synthesises the essential amino acid from glutamate and ammonium. The GS gene can be used as selectable marker when methionine sulphoximine is added to the medium which inhibits endogenous GS function. Thus, only cells with additional GS will survive (Birch & Racher, 2006; Lonza, 2005).

When choosing an expression system, also the host cell is of great importance. Especially for a highly glycosylated protein like gp120 the host cell should be capable of producing adequate glycosylation patterns which are important for the immunogenicity of the protein. Several comparisons showed that recombinant gp120 derived from baculovirus expression systems failed e.g. in humoral immunogenicity tests while CHO-derived gp120 showed potent neutralizing ability (Jeffs et al., 2006).
Thus, the CHO expression system seems to be a rational choice.

2.4. Affinity Chromatography

The purification of certain proteins out of a fermentation broth always plays a major role in the production of pharmaceutical proteins. As high purity is required for such proteins, focus is placed on methods capable of achieving significant purification in one single step. Thus, affinity separation methods are a logical choice (Yarmush et al., 1992).

In affinity chromatography, a specific ligand is coupled to a chromatography matrix. The reversible interaction of the ligand with a protein (or group of proteins) is used to separate the protein(s) of interest from other proteins present in the sample. The high-selectivity, high-resolution and high-capacity allow for purification levels in the order of several thousand-fold and target proteins can be collected in a purified and concentrated form (GE Healthcare, 2007; Azarkan et al., 2007).

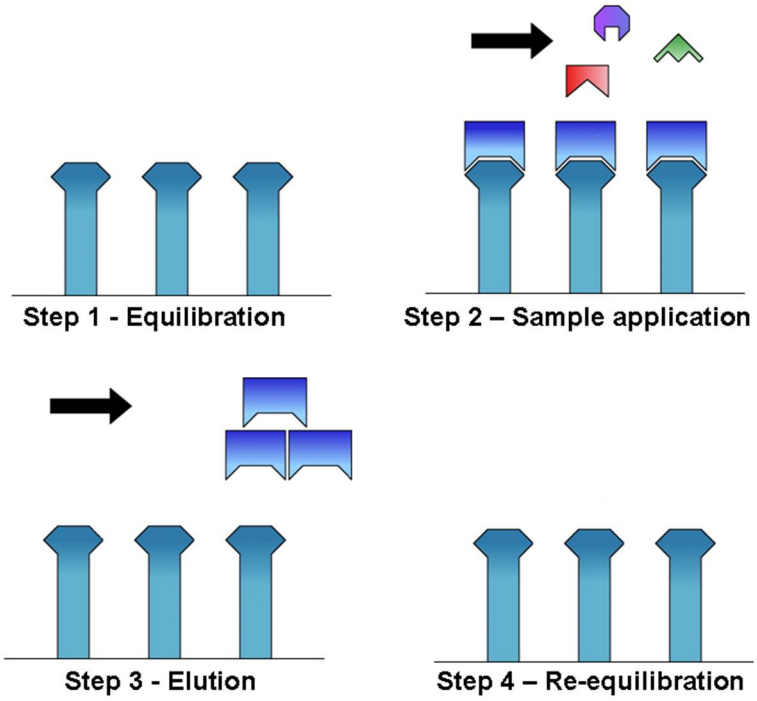


Figure 2: Depiction of steps during affinity chromatography

Figure 2 shows the individual steps taking place during a typical affinity chromatography.

Step 1: The ligand is coupled to a chromatography matrix and the resulting affinity medium is equilibrated in binding buffer. Some typical frequently used biological interactions are:

- Antibody ↔ antigen
- Enzyme ↔ substrate
- Lectin ↔ glycoprotein
- Hormone, vitamin ↔ receptor, carrier protein

For successful affinity purification, covalent attachment of the ligand to the chromatography matrix must be possible while the specific binding affinity for the target molecule must be retained.

Step 2: Sample application is performed using conditions which enhance the binding of the target protein. Simultaneously, unbound material is washed away.

Step 3: After sample application is completed and all unbound material is washed out, conditions are changed to enhance elution of the bound molecules. Elution can be performed for example by changing the pH or ionic strength, using chaotropic salts like thiocyanate, denaturants like urea or guanidine hydrochloride or organic solvents like ethylene glycol. For specific elution also competitive ligands can be used. However, most gently elution conditions should be chosen to retain the biological activity of target protein and ligand.

Step 4: After elution is completed, the affinity medium is re-equilibrated with binding buffer or stored in storage solution (e.g. 20 % ethanol or 0.05 % NaN_3).
(GE Healthcare, 2007; Yarmush et al., 1992)

Figure 3 shows a typical chromatogram obtained during an affinity chromatography.

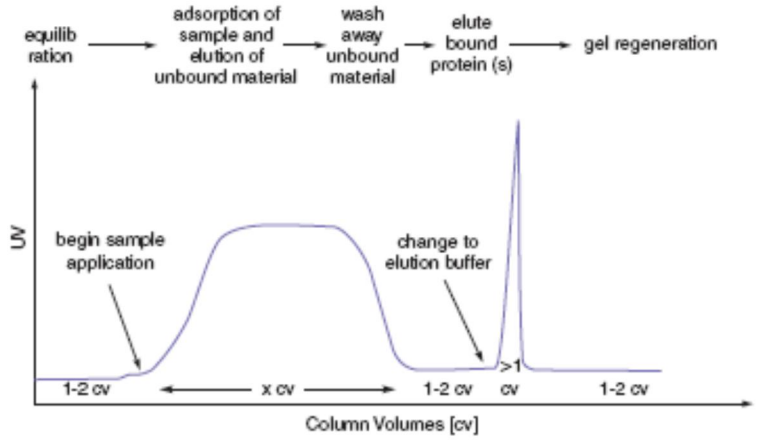


Figure 3: Typical affinity chromatography (GE Healthcare, 2007)

2.5. Analytical Methods

2.5.1. Enzyme-Linked Immunosorbent Assay (ELISA)

An enzyme-linked immunosorbent assay (ELISA) is a testing method that uses the highly specific antibody-antigen interaction, which is detected by an enzyme catalysed colour reaction. These interactions can be used for quantitative or qualitative determination of antigens or antibodies. In the process one component is fixed to a solid support resulting in the ability to bind the other component out of the sample solution. Undesired proteins are removed through washing and the antibody and antigen respectively may be detected. Verification of this complex is done by means of either a radioactive labelled (radio-immuno assay, RIA) or an enzyme-linked secondary antibody (ELISA). Most commonly used enzymes are horseradish peroxidase (HRP) or phosphatase. P-Nitrophenylphosphate (for phosphatase) and o-phenylenediamine (for peroxidase) are widely used substrates (Beck-Sickinger & Aicher, 1998).

Several forms of ELISA exist. For a competitive ELISA the antibody is adsorbed to a supporting material. A mixture of test sample containing antigen plus enzyme labelled antigen is added. Both antigens compete for the antibody's binding site. Proper substrate is added and the difference in signals from the mixture compared with pure labelled antigen specifies the amount of not-labelled antigen in the mixture. Another method for determining an antigen is the sandwich technique. Again an antibody specific for the antigen to be detected is adsorbed to a solid support. Test sample is added and antigen is bound by the antibody. A second antibody that is also specific for the antigen and labelled with an enzyme is added. Finally adequate substrate is added again. ELISA is also a suitable method to detect antibodies. Therefore the antigen is immobilized and bound antibody is detected with an enzyme labelled antiglobulin. Obviously, it is necessary that test vessels are washed after each step to remove unbound material (Voller et al., 1978).

In this work a slightly modified sandwich-ELISA was applied for detection of gp120 in solutions (see figure 4). Lectin from *Galanthus nivalis* (snowdrop) which is capable to bind carbohydrates was used to bind gp120 in the individual samples (DeHoff et al., 2009).

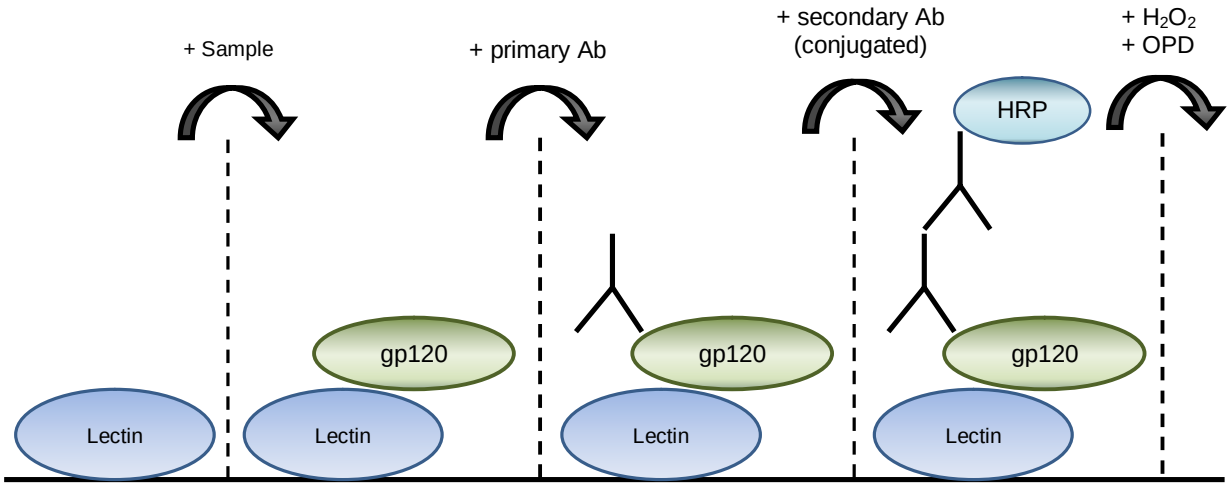


Figure 4: Schematic setup and procedure of ELISA

Adsorption of the ligand to be immobilized to the polystyrene surface of the used microtiter plates is caused by the special characteristics these plates display on their surface. It presents a fine mesh of hydrophobic and hydrophilic binding sites which facilitate adsorption of hydrophilic molecules by competition with water molecules for binding the macromolecules through hydrogen bonds (NUNC).

The detection antibody or primary antibody is directed against the analyte and can be conjugated with one of the enzymes mentioned above (Beck-Sickinger & Aicher, 1998).
However, when several ELISAs with different analytes and detection antibodies are performed, it may be more economical to use a conjugated antibody or secondary antibody that is directed against the species of the detection antibody. If all used primary antibodies origin from the same species (e.g. mouse) it is possible to use the secondary (enzyme conjugated) antibody (e.g. goat-anti-mouse) for all assays.

The addition of the enzyme-specific substrate allows the detection of the analyte via a colouring reaction that is triggered by the enzyme.
For example, HRP catalyses the reduction of H_2O_2 and simultaneous oxidation of o-phenylenediamine (OPD). OPD is oxidized to 2,3-diaminophenazine which gives a brownish colour (Tarcha et al., 1987).
In microplate reader the staining can be measured. Standard values are used to create a standard curve which in turn allows calculation of analyte concentrations.

Other ELISA methods which were applied in the course of this work are described in section 3.2.3.1.

2.5.2. Sodium Dodecyl Sulphate – Polyacrylamide Gel Electrophoresis (SDS-PAGE)

During electrophoresis, charged particles move to the electrode with opposite charge in a field of direct current. The definition includes three different types of electrophoretical separation methods: electrophoresis or zonal electrophoresis (ZE), isotachophoresis (ITP) and isoelectric focusing (IEF).
A homogeneous buffer system is used for electrophoresis providing a constant pH across the whole separation distance. The sample components separate due to different electrophoretic mobility.
During isotachophoresis, the components move with the same velocities between a fast leading electrolyte and a slow terminating electrolyte. The components form a stack in which the components with highest mobility directly follow the leading electrolyte whereas those with low mobility move directly in front of the terminating electrolyte. All other components move in the order of decreasing mobilities between these components.
Isoelectric focusing requires a pH gradient. It can only be used for amphoteric compounds i.e. proteins and peptides (containing both, positive and negative charges) and depends on the net charge of the individual proteins which is built by the sum of positive and negative charges of the aminoacids with charged side chains. While carboxyl groups are charged negatively at high pH they are neutral at low pH. In contrast, amino and imidazole groups are neutral at high pH and charged positively at low pH. Thus, the proteins move in the electric field until they reach the pH at which the positive charges are equal to the negative ones resulting in a neutral net charge (isoelectric point) (Stransky & Eckstein, 1998).

Polyacrylamide gels are restrictive i.e. the electrophoretic mobilities depend on both net charge and molecule radius. In SDS-PAGE, the molecules are separated only depending on the molecule size as the molecule's intrinsic charge is superimposed effectively by sodium dodecyl sulphate (SDS). This results in micelles with constant net charge per mass unit – 1.4 g SDS bind to 1 g protein. Different molecule forms are compensated by cleaving hydrogen bonds with reducing agents such as dithiothreitol (DTT) (Stransky & Eckstein, 1998).

The three principles are depicted in Figure 5.

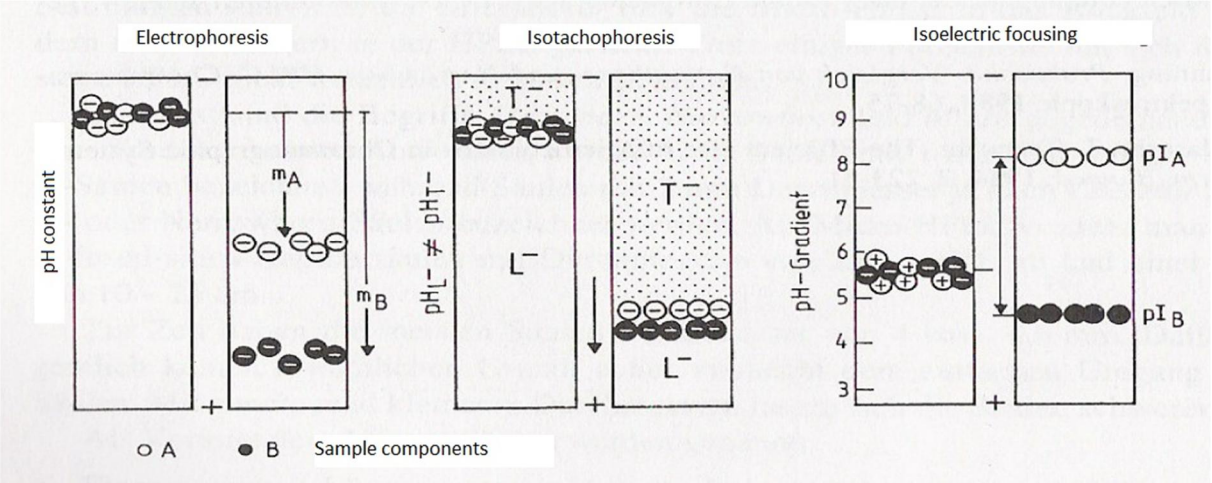


Figure 5: Principles of electrophoresis (Stransky & Eckstein, 1998)

2.5.3. Western Blot

In a Western Blot the electrophoretically separated proteins are transferred on a suitable membrane and detected using immunological methods. Transfer of the proteins to the membrane concentrates the molecules which were previously distributed in the gel on the membrane's surface where detection is easier. Western Blot allows the detection of non-labelled antigens in complex mixtures and determination of their relative molecular weight and quantity (Klein & Müller, 1998).

The transfer from the gel onto the membrane is done in an electrical field. After completion of the transfer unspecific binding sites on the membrane are blocked with a protein solution e.g. dissolved skim milk powder, bovine serum albumin or similar to enhance the signal-to-background ratio. A specific antibody is then used to detect the antigen on the membrane. This antibody is either directly labelled or a labelled secondary antibody which is directed against the detection antibody is used. Labelling of the antibodies can be done with enzymes, colloidal gold or radioactive. Enzyme-labelled antibodies can be detected by colouring reactions or chemiluminescence (Klein & Müller, 1998).

3. Materials and Methods

3.1. Materials

3.1.1. Chemicals

Table 1: Used chemicals

Chemical	Supplier/Manufacturer Cat.No.
5-bromo-4-chloro-3-indolyl phosphate	Fluka 16670
Acetic acid (glacial)	Merck 1.00063
Bovine serum albumin (BSA)	Sigma A7906
Citric acid monohydrate	Merck 1.00244
Coulter counter buffer (ISOTON Diluent)	Beckman Coulter 8546719
Coomassie Blue R250	Fluka 27816
Dimethylformamide	Sigma D4551
Disodium hydrogen phosphate dihydrate	Merck 1.06580
DMEM/HAM's F12	Biochrom F4815
Ethanol	Australco P20501
Ethanolamine	Sigma E0135
Ferric(III)citrate	Sigma F3388
Formaldehyde	Merck 1.04002
Glutaraldehyde	Fluka 49629
Glycine	Merck 5.00190
GS Supplement	SAFC 58672C
Hydrogen peroxide 35 %	Applichem APPCA0466.5000
L-Ascorbic acid	Sigma A4544
L-Methionine sulfoximine (MSX)	Sigma M5379
Magnesium chloride (hexahydrate)	Merck 1.05833
Nitro-blue tetrazolium	Fluka 74030
NuPAGE® LDS sample buffer	Invitrogen NP0007
NuPAGE® MES SDS running buffer	Invitrogen NP0002
NuPAGE® transfer buffer	Invitrogen NP0006
NuPAGE® Tris-Acetate SDS running buffer	Invitrogen LA0041

Chemical	Supplier/Manufacturer Cat.No.
o-Phenylenediamine dihydrochloride	Sigma P1526
Pluronic F68	Sigma P1300
Polyvinylpyrrolidone	Sigma PVP40-T
Potassium chloride	Merck 1.04936
Potassium dihydrogen phosphate	Merck 1.04873
Silver nitrate	Merck 1.01512
Skim milk powder	Fluka 70166
Sodium acetate (anhydrous)	Merck 1.06268
Sodium bicarbonate	Merck 1.06329
Sodium butyrate	Merck 8.17500
Sodium carbonate	Merck 1.06392
Sodium chloride	Merck 1.06404
Sodium selenite	Sigma S5261
Sodium thiosulphate pentahydrate	Merck 1.06516
Soy peptone	Quest 51558TT
Sulfuric acid	Merck 1.00716
Titriplex III	Merck 1.08418
Tris	Merck 1.08386
Trypan blue	Sigma T6146
Tween 20	Sigma P7949

3.1.2. Immunochemicals

Table 2: Proteins/antibodies used in ELISA and Western Blot

Protein/Antibody	Supplier/Manufacturer Cat.No.
2G12	Polymun
B12	Polymun
HRP-Goat anti-Human IgG (Gamma)	Zymed 62-8420
goat anti-Human IgG (ɣ-chain) – AP	Sigma A3187
Lectin from *Galanthus nivalis*	Sigma L8275

3.2. Methods

3.2.1. Cultivation of CHO cells

3.2.1.1. Composition of Culture Medium

Culture Medium for CHO cells consisted of following components:
500 ml DMEM/HAM's F12, 5 ml protein-free (PF) additive, 10 ml soy-pepton/UF, 5 ml Pluronic F68, 10 ml GS supplement and 4 ml MSX.

3.2.1.2. Routine Cultivation

CHO cells were cultivated in 100 ml spinner flasks with a cell-suspension volume of 50 ml. Every 3 or 4 days a 5 ml sample was taken. 1 ml of this sample was used for determining viability by a trypan blue exclusion assay. Therefore 200 µl trypan blue were added and approximately 20 µl of the mixture were pipetted into a Neubauer hemocytometer. 4 or 8 large squares were counted to achieve a total sum of counted cells of at least 200. Viability was calculated by division of living cells through total cell number. The residual 4 ml were centrifuged for 7 min at 350 g and the supernatant was discarded. 1 ml Coulter counter buffer was added and incubated at room temperature for at least 30 min. Cell number was determined with a Coulter counter. For further cultivation cell density was adjusted to approximately 1 x 10^5 cells/ml and spinner flasks were incubated at 37 ± 2 °C.

3.2.1.3. Batch Cultivation

Batch cultivation was done in 500 ml spinner flasks with cell-suspension volumes of 300 - 500 ml. Batches were started with a cell density of approximately 1 x 10^5 cells/ml. Various amounts of sodium butyrate (to an end concentration of 0.1 - 0.4 mM) were added, each flask was fumigated with 120 ml CO_2 and flasks were incubated at 37 ± 2 °C. Viability and cell number was determined every 1 – 3 days as described above. Additionally, the supernatant was not discarded after centrifugation but collected for quantification of the gp120 yields. When viability fell below 50 % the cell broth was transferred into centrifuge tubes and centrifuged for 10 minutes at approx. 270 g. Supernatant was transferred into sterile roller bottles and stored at ≤ -20 °C until further processing.

3.2.2. Downstream Processing

3.2.2.1. Ultra/Diafiltration

To reduce the working volume and to exchange buffer for chromatography, an ultra/diafiltration of the cell culture supernatant was performed using a Millipore Labscale™ TFF System equipped with a Millipore Pellicon 50 cm^2 membrane with a pore size of 100 kDa. Cell-free supernatant was processed in two portions of 1000 ml each. The system's cylinder was therefore filled with 500 ml of supernatant and ultrafiltration was started. Inlet pressure was adjusted to approx. 2 bar and retentate pressure was adjusted to approx. 0.5 bar. When necessary supernatant was replenished until the complete 1000 ml were used. Filtration was performed until 50 ml of retentate were left. Then, diafiltration was started by adding 50 ml of buffer (20 mM Tris / 0.5 M NaCl, pH 8.3). Filtration was continued until 50 ml of retentate remained in the cylinder. The procedure was repeated for a total of 7 buffer exchanges.
Retentate was then aseptically filtered using a 0.2 µm funnel filter unit and stored at ≤ -20 °C.

3.2.2.2. Preparation of columns for use in affinity chromatography

Columns with both antibodies, B12 and 2G12, were prepared using the same procedure.
The antibodies were transferred into coupling buffer (0.1 M $NaHCO_3$ / 0.5 M NaCl, pH 8.3) via a GE Healthcare PD10 buffer exchange column according to the manufacturer's instructions.
Approximately 285 mg of Toyopearl AF-Tresyl-650 resin were swollen in approximately 5 ml RO-water. After sedimentation of the chromatography matrix, the supernatant was carefully removed by pipetting. Afterwards the resin was re-suspended in coupling buffer for washing. Two of these wash steps with coupling buffer were performed. The remaining supernatant was removed using a pipette and antibody solution (in coupling buffer) was added to achieve a total amount of 3 mg. The slurry was incubated on a shaker for 4 hours at room temperature. Supernatant was then removed and collected and resin was washed twice with coupling buffer. Supernatants of the wash steps were also collected for later determination of the binding ratio.
Approximately 5 ml of 1 M ethanolamine were added and slurry was incubated at 5 ± 3 °C over night to block remaining free binding sites.
On the next day, the resin was re-suspended, transferred into a pre-wetted HR 5/5 column (GE Healthcare) and packed at a flow rate of 0.9 ml/min using an Äkta Explorer chromatography system (GE Healthcare) and coupling buffer. Until not stated different, a

flow rate of 0.8 ml/min was used. 1 M ethanolamine was pumped across the column for 1 hour. 0.1 M glycine / 0.5 M NaCl pH 9.0 was pumped across the column until alkaline pH was detected at column outlet and then continued for 15 minutes. Afterwards, 0.1 M glycine / 0.5 M NaCl pH 4.0 was pumped across the column until acidic pH was detected at column outlet and then continued for 15 minutes. These two steps were repeated for a total of three times. Afterwards the column was equilibrated with 20 mM Tris / 0.5 M NaCl pH 8.3.
Afterwards, 1 % polyvinylpyrrolidone (PVP) was pumped across the column for 1 hour. PVP was then washed out using 20 mM Tris / 0.5 M NaCl pH 8.3. Column was afterwards brought to neutral pH using 0.1 M glycine / 0.1 M NaCl pH 7.5 and stored in 0.05 % sodium azide.

For calculation of the ligand binding ratio, the antibody concentrations in the supernatants obtained during coupling were determined by measuring the optical density at 280 nm. The initial amount was set to 100 % and the amounts remaining in the supernatants were subtracted.

3.2.2.3. Affinity Chromatography - Purification Procedure

Except the elution buffers which were also used for regeneration of the column, the running conditions were the same for both columns. 2 M $MgCl_2$ / 0.05 M glycine pH 6.0 was used as elution buffer for the 2G12 column, 0.1 M glycine pH 3.0 was used for the B12 column.
The particular column was regenerated with elution buffer and equilibrated with 20 mM Tris / 0.5 M NaCl pH 8.3 at a flow rate of 0.8 ml/min. If not stated different, a flow rate of 0.1 ml/min was used for the following steps. This is equivalent to a linear velocity of 30 cm/h and results in a residence time of more than 10 minutes as all used columns have more than 1 ml of volume.
Sample was applied followed by washing with 20 mM Tris / 0.5 M NaCl pH 8.3. Flow-through was collected as wash 1 (FT + W1). For determination of the dynamic binding capacity, collection was performed in fractions of approx. 1 ml each. Afterwards column was washed with 0.1 M glycine / 0.1 M NaCl pH 7.5. Flow-through was collected as wash 2 (W2). Column was then washed with 0.05 M glycine pH 7.5 and flow-through was collected as wash 3 (W3).
After washing was completed the adequate elution buffer was applied and eluate was collected.

3.2.3. Analytical Procedures

3.2.3.1. ELISA

3.2.3.1.1. Composition of buffers and solutions

Coating buffer

8.4 g $NaHCO_3$

4.2 g Na_2CO_3

ad 1000 ml RO-water

pH 9.5 – 9.8

Wash buffer (PBS-T)

1.15 g $Na_2HPO_4 * 2\ H_2O$

0.2 g KH_2PO_4

0.2 g KCl

8.0 g NaCl

1.0 ml Tween 20

ad 1000 ml RO-water

pH 7.2 – 7.4

1 % BSA (dilution buffer)

2.0 g BSA

ad 200 ml PBS-T

Staining buffer

7.3 g citric acid * H_2O

11.86 g $Na_2HPO_4 * H_2O$

ad 1000 ml RO-water

pH 4.8 – 5.0

Ortho-phenylenediamine (OPD) stock solution

10 g OPD

ad 100 ml staining buffer

Staining solution

100 µl OPD stock solution

7 µl H_2O_2

in 10 ml staining buffer

3.2.3.1.2. Quantification of gp120 in solutions

Microtiter plates (Nunc Maxisorp) were coated with 1 µg/ml lectin from *Galanthus nivalis* in coating buffer (100 µl/well) and incubated at 5 ± 3 °C for at least 8 hours or at room temperature (RT) for at least 2 hours.

Purified gp120 was used as standard in a concentration of 300 ng/ml. Samples were diluted in 1 % BSA to achieve a concentration of approximately 300 ng/ml. A low adsorption plate was used to generate an 1.5^8 dilution series. Therefore, 300 µl of standard and samples were pipetted into row H of the dilution plate using well H1 as blank filled with 300 µl of 1 % BSA. Rows A – G were filled with 100 µl of 1 % BSA. 200 µl from row H were transferred into row G and mixed by pipetting. Afterwards 200 µl of row G were transferred into row F and again mixed by pipetting. This procedure was repeated across the whole plate obtaining the lowest concentration in row A.

The lectin-coated plates were washed 4 times with PBS-T using a plate washer (Biotek). Remaining liquid was removed by vigorous banging on paper cloth and 100 µl of 1 % BSA were pipetted into each well and incubated on a plate shaker for 1 hour at RT to block any remaining unspecific binding sites.

After completion of the blocking step, the plate was washed 4 times and 50 µl from each well of the dilution plate were transferred to the respective wells of the measuring plate. The measuring plate was then incubated on the shaker for 1 hour at room temperature.

After the incubation the plate was washed 4 times and 50 µl of 2G12 antibody diluted in 1 % BSA to ~1 µg/ml was added to each well followed by 1 hour incubation at room temperature on the shaker.

Subsequently, the plate was washed 4 times again and 50 µl of horseradish peroxidase conjugated goat anti-human IgG (γ-specific) antibody diluted 1:5000 in 1 % BSA were added to each well and incubated for 1 hour at room temperature on a shaker.

Finally the plate was washed 4 times and 100 µl/well of staining solution were added. Staining was allowed to take place until the desired intensity (visible staining of lowest standard) was achieved followed by adding 100 µl/well of 25 % H_2SO_4.

Plates were then put in the plate reader (Synergy 2, Biotek) and read at 492 nm using 620 nm as reference. Standard curve was generated automatically by the Gen5 software using a 4-parameter fit. Gp120 concentration in the samples was determined automatically using the standard curve.

3.2.3.1.3. Stability of 2G12 in low pH buffer and $MgCl_2$ buffer

To investigate the binding ability of 2G12 after exposure to low pH, the antibody solution was mixed with 0.1 M glycine pH 2.5 to obtain antibody in solutions of pH 3.0 and pH 2.6. The antibody solutions where then further diluted with 1 % BSA to a final concentration of

200 ng/ml each. Untreated antibody was diluted with 1 % BSA to 200 ng/ml as well and used as control.
280 µl of each solution were then transferred onto a gp120-coated MaxiSorp microtiter plate and a 2^8 dilution series was generated by 7 consecutive 1:2 dilutions with 1 % BSA. The plate was afterwards incubated on a shaker for 1 hour at RT. Subsequently, the plate was washed 4 times with PBS-T and further analysis was performed as described in 3.2.3.1.2. using HRP-conjugated goat anti-human IgG. A curve for each sample was generated and the three curves were compared.

In a second attempt the binding ability of 2G12 after exposure to a buffer containing 2 M $MgCl_2$ was evaluated. Therefore, 2G12 was diluted 1:5 with 2.5 M $MgCl_2$ / 0.0625 M glycine pH 6.0 and incubated 1 h at RT. Afterwards the solution was further diluted with 1 % BSA to obtain a final concentration of 200 ng/ml and analysed as described above using untreated antibody as control.

3.2.3.1.4. Stability of B12 in low pH buffer and $MgCl_2$ buffer

As for 2G12 the binding ability of B12 after exposure to low pH and 2 M $MgCl_2$ was investigated. Therefore, the antibody solution was mixed with 0.1 M glycine pH 2.0 to obtain a solution with pH 2.5 and diluted 1:5 with 2.5 M $MgCl_2$ / 0.0625 M glycine pH 6.0. The solutions were incubated 1 h at RT and further analysed as described in 3.2.3.3 using untreated antibody as control.

3.2.3.1.5. Binding stability of B12 in buffers of different pH

To test the binding stability of B12 when exposed to different pH-values, a microtiter plate was coated over night with gp160 at 4 °C. Afterwards, the plate was washed and 100 µl/well of a 450 ng/ml B12-solution were applied and incubated for 1 hour at room temperature. After an additional washing step, PBS adjusted to different pH-values (2.0 – 8.5; in 0.5 steps) was applied to the plate using 4 wells per pH with PBS pH 7.37 as control. The PBS was incubated at room temperature for 1 hour. Subsequently, plate was washed, HRP-conjugated goat anti-human IgG (diluted 1:5000 in 1 % BSA) was applied (100 µl/well) and incubated for 1 hour at room temperature. Staining was performed as described in 3.2.3.1.2. and mean ΔOD values of the individual pH-values were compared to that of the control.

3.2.3.1.6. Determination of host cell protein contents

Host cell protein contents of an eluate obtained with a 2G12 column and one eluate obtained with a B12 column were analysed using a commercial available ELISA kit (Cygnus, F015) according to the high sensitivity protocol from the product insert.

A 2^8 dilution series of the 100 ng/ml standard included in the kit was used for generation of a standard curve. Samples were analysed in dilutions of 1:2, 1:4, 1:8 and 1:16.
100 µl/well of each standard and each sample concentration (in duplicates) were transferred into the pre-coated microtiter strips from the kit and incubated 1 hour at room temperature. After incubation was completed, the plate was washed twice with washing buffer included in the kit and 100 µl/well of anti-CHO:HRP conjugate was applied to each well and incubated for 2 hours at room temperature. Afterwards the plate was washed 4 times and 100 µl/well of pNPP substrate were added and incubated 30 minutes without shaking and protected from light. Staining reaction was afterwards stopped with stop solution and plate was read at 450/650 nm blanking on the zero standard.

3.2.3.1.7. Quantification of human IgG in solutions

Microtiter plates (Nunc Maxisorp) were coated with goat-anti-human IgG (gamma-chain specific) in coating buffer (100 µl/well) and incubated at 5 ± 3 °C for at least 8 hours or at room temperature (RT) for at least 2 hours.
The internal 2G12 was used as standard in a concentration of 200 ng/ml for samples containing 2G12 while human IgG (Sigma, I4506) in a concentration of 200 ng/ml was used for quantification of B12. Samples were diluted in 1 % BSA to achieve a concentration of ~200 ng/ml or used undiluted if estimated antibody concentration was lower than 200 ng/ml. A low adsorption plate was used to generate a 2^8 dilution series. Therefore, 280 µl of standard and samples were pipetted into row H of the dilution plate using well H1 as blank filled with 280 µl of 1 % BSA. Rows A – G were filled with 140 µl of 1 % BSA. 140 µl from row H were transferred into row G and mixed by pipetting. Afterwards 140 µl of row G were transferred into row F and again mixed by pipetting. This procedure was repeated across the whole plate obtaining the lowest concentration in row A.
The coated plates were washed 4 times with PBS-T using a plate washer (Biotek). Remaining liquid was removed by vigorous banging on paper cloth and 50 µl from each well of the dilution plate were transferred to the respective wells of the measuring plate. The measuring plate was then incubated on the shaker for 1 hour at room temperature.
After the incubation the plate was washed 4 times and 50 µl of horse radish peroxidase-conjugated goat-anti-human IgG (gamma-chain specific) antibody diluted in 1 % BSA to ~1 µg/ml was added to each well followed by 1 hour incubation at room temperature on the shaker.
The plate was washed 4 times and 100 µl/well of staining solution were added. Staining was allowed to take place until the desired intensity (visible staining of lowest standard) was achieved followed by adding 100 µl/well of 25% H_2SO_4.

Plates were then put in the plate reader (Synergy 2, Biotek) and read at 492 nm using 620 nm as reference. Standard curve was generated automatically by the Gen5 software using a 4-parameter fit. Antibody concentrations in the samples were determined automatically using the respective standard curves.

3.2.3.2. SDS-PAGE & Western Blot

SDS-PAGE was performed on a Novex system using a 3-8 % Tris-Acetate gel and a NuPAGE Tris-Acetate SDS running buffer. 18 µl of sample were mixed with 6 µl of NuPAGE LDS sample buffer and 20 µl were applied onto the gel. A prestained marker was used as reference. Separation was conducted at a voltage of 150 V for approximately 1 hour.
Visualization of bands was done by silver staining according to Heukeshoven. Therefore the gel was removed from the cassette and covered with fixing solution (40 % ethanol, 10 % acetic acid) and incubated for at least 20 minutes. Solution was decanted and the gel was incubated for approximately 40 minutes in incubation solution (30 % ethanol, 6.8 % sodium acetate, 0.125 % glutaraldehyde, 0.127 % sodium thiosulphate). Afterwards the gel was washed 3 times for at least 5 minutes with RO water. The gel was then covered with silver solution (0.1 % silver nitrate, 0.007 % formaldehyde) and incubated for ~ 20 minutes. After decantation of the silver solution, the gel was incubated in developing solution (2.5 % sodium carbonate, 0.0035 % formaldehyde) until the desired staining intensity was achieved. Immediately, afterwards incubation solution was decanted and gel was covered with stop solution (0.73 % Titriplex III) for at least 10 minutes.

For Western Blot, an SDS-PAGE was performed on a Novex System using a 4 -12 % Bis-Tris gel and a NuPAGE MES SDS running buffer. 18 µl of sample were mixed with 6 µl of NuPAGE LDS sample buffer and 20 µl were applied onto the gel. Each sample was applied twice onto the gel. A prestained marker was used as reference. Separation was conducted at a voltage of 200 V for approximately 45 minutes. After completion of the separation procedure the gel was cut into two pieces. One part was stained with Coomassie while the other part was used for the Western Blot.
Coomassie staining was performed by incubating the gel in a staining solution (1 % Coomassie Blue R 250, 40 % ethanol, 10 % acetic acid in HQ-water) for 30 minutes. The gel was afterwards destained with 10 % ethanol, 7.5 % acetic acid in HQ-water until the desired band intensity was achieved.

Blotting was performed on a Novex system using a nitrocellulose blot membrane and a NuPAGE transfer buffer containing 10 % ethanol. The blot procedure was conducted at 25 V

for 1 – 1.5 hours. After completion of the transfer the blot membrane was incubated for approx. 1 hour in 3 % skim milk powder (SMP) in PBS-T to block unspecific binding sites. The membrane was then briefly washed in PBS-T and incubated in the first antibody solution (either 1.5 µg/ml 2G12 or 3.9 µg/ml B12 in 1 % SMP in PBS-T) for 1 hour. Afterwards the membrane was washed 3 times in PBS-T for a total of at least 15 minutes. Subsequently, it was incubated in the second antibody solution (anti-human IgG (γ-chain specific) conjugated with alkaline phosphatase in 1 % SMP in PBS-T) for 1 hour. The membrane was then washed 3 times in PBS-T for a total of at least 10 minutes followed by two washes of 5 minutes each in staining buffer (0.1 M Tris, 0.1 M NaCl, 5 mM $MgCl_2$ * 6 H_2O). The blot was developed in staining solution composed of 1 µl BCIP stock solution (70 mg 5-bromo-4-chloro-3-indolyl phosphate in 1000 µl dimethylformamide) and 1 µl NBT stock solution (70 mg nitro-blue tetrazolium + 700 µl dimethylformamide + 300 µl HQ-water) per ml of staining buffer until the desired intensity was obtained. The staining process was stopped by washing the blot with HQ-water.

4. Results and Discussion

4.1. Influence of sodium butyrate in cell cultivation

To investigate the influence of sodium butyrate on cell growth, viability and most important gp120 yield at the end of the batch, a batch using 5 spinner vessels was started. Sodium butyrate was added in following concentrations: 0 mM (reference = K), 0.1 mM, 0.2 mM, 0.3 mM and 0.4 mM. Batch culture was performed as described in 3.2.1.3. Figure 6 shows progress of cell count and viability in the five spinner vessels. The spinner vessel containing 0.2 mM sodium butyrate was found to be contaminated on day 10. As this sodium butyrate concentration was bracketed by two higher and two lower concentrations no further experiments at 0.2 mM sodium butyrate were made and the spinner vessel concerned was excluded from further processing.

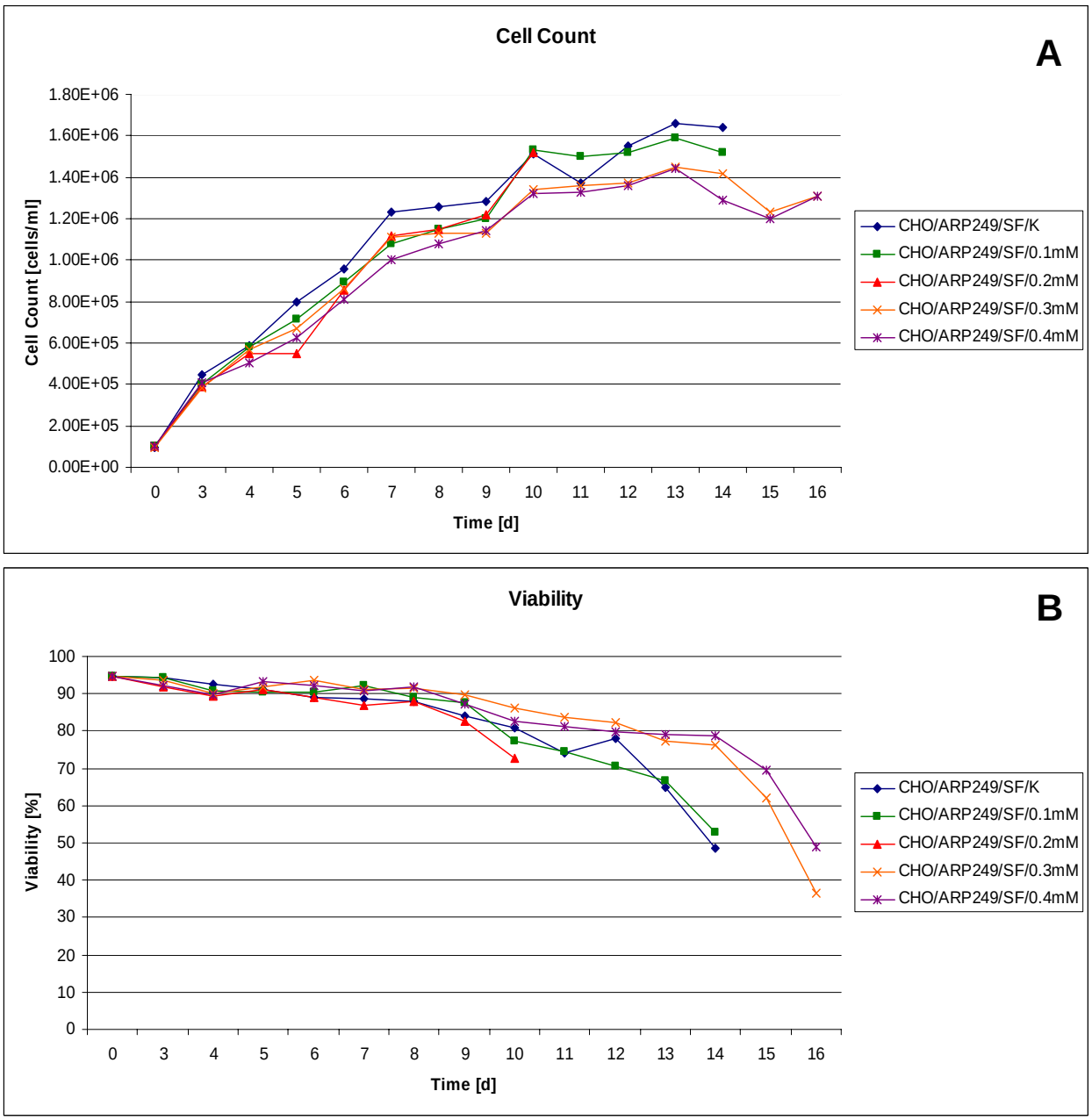


Figure 6: Influence of various sodium butyrate concentrations on cell growth and viability. (A) shows the cell count over the cultivation period while (B) is a plot of viability versus time.

Figure 7 shows the gp120 concentrations in the culture supernatant at the end of the batch cultivation as determined by ELISA. As mentioned above, the spinner vessel containing a concentration of 0.2 mM sodium butyrate was contaminated and excluded from further experiments. The data above show that sodium butyrate in fact has an influence on cell growth and viability. While cell growth seems to be slowed, viability is maintained at higher levels (compare day 14). Also the calculated specific productivity based on the viable cell densities shows a response to the applied sodium butyrate concentration (see Figure 7). The mean specific productivity in spinner vessels with 0.3 mM and 0.4 mM added sodium butyrate is higher than in the control and 0.1 mM sodium butyrate vessel.

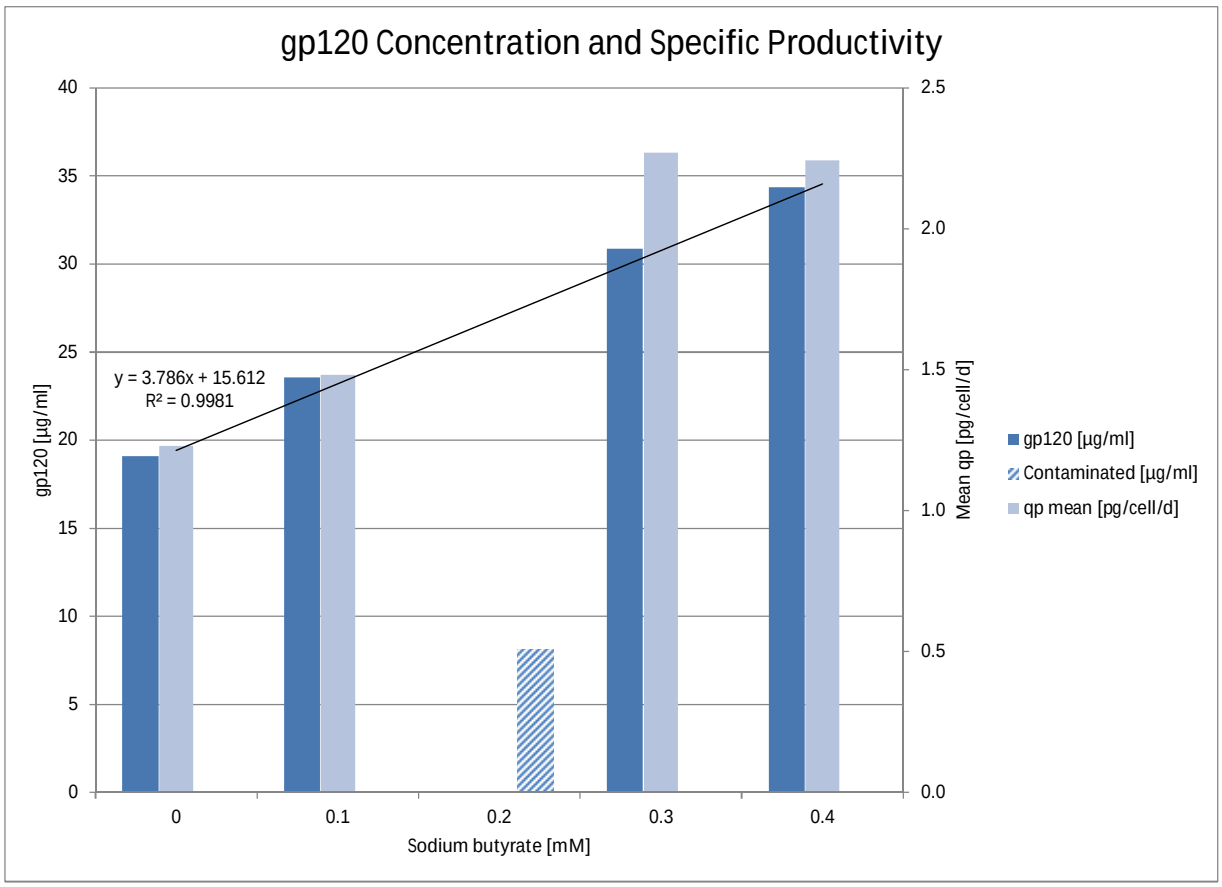


Figure 7: Final gp120 concentration at the end of the batch cultivation and mean specific productivity using different sodium butyrate concentrations. Result shaded in grey was obtained in the contaminated spinner vessel.

Higher viability allows an extension of the cultivation period while higher specific productivity improves the final concentration. In detail, the gp120 concentration in 0.4 mM sodium butyrate batch was 34.37 µg/ml whereas the concentration in the reference batch without sodium butyrate was 19.09 µg/ml which corresponds to an increase of 80 %. As can be seen in Figure 7 above, a high correlation of the applied sodium butyrate concentration and the gp120 concentration at the end of the batch was obtained. Application of a linear regression results in a calculated coefficient of determination of 0.9981. As this value indicates a high linearity in the investigated range of sodium butyrate concentration, a higher concentration might even result in further increase of gp120 concentration at the end of the batch cultivation. In synthesis of recombinant human prolactin and production of recombinant human interferon-β-1a, a sodium butyrate concentration of 1 mM was described to result in highest product yields (Rodrigues Goulart et al., 2010, Oh et al., 2005). Thus, this concentration might also be beneficial in production of recombinant gp120.

4.2. Downstream Processing Development

The first step was the coupling of antibodies to a chromatography matrix. In previous purification processes the use of Toyopearl AF-Tresyl-650 (Tosoh Biosciences GmbH, Germany) gave good results (data not shown here). In total 6 columns were prepared, which are shown in Table 3, using the antibodies 2G12 and B12 for coupling.

Antibody	Column No.	Used Ligand [mg]	Coupled Ligand [mg]	Ligand Density [mg/ml]	Degree of Coupling [%]	Column Volume [ml]
2G12	1	2.63	0.71	0.53	27.0	1.34
2G12	2	4.38	3.22	2.37	73.7	1.35
2G12	3	7.27	5.34	4.77	73.5	1.12
2G12	4	3.36	2.67	2.12	79.5	1.26
2G12	5	3.19	2.76	2.34	86.5	1.18
B12	6	3.29	3.15	2.76	95.7	1.14

Table 3: Comparison of Coupling Ratios

With a coupling ratio of 27 %, the first attempt of coupling antibody 2G12 to the column appeared to be inefficient. This was most likely caused by an operator error when adjusting pH of the coupling buffer as Tris was used instead of NaOH. Due to this mistake, binding sites of the matrix which would usually be occupied by antibody were blocked by Tris causing the small degree of coupling.

In the normal coupling procedure, unoccupied binding sites on the matrix are blocked by ethanolamine during the incubation steps (overnight at 5 ± 3 °C and 1 hour after column packing). This small molecule displays a hydroxyl group on the free side. Tris displays three of the same groups. Thus, interaction of the blocking agent ethanolamine and the accidentally added Tris with gp120 is considered comparable and not having a significant impact on comparability of the different columns.

All further coupling procedures were satisfactorily efficient with coupling ratios of 73.5 – 86.5 % for 2G12 and even 95.7 % for B12. As no comparable values for coupling efficiency could be found in literature, the obtained results are considered sufficient.

In the next step, the 2G12 column was used for purification of gp120 out of ultradiafiltered cell culture supernatant. In the first experiments 0.1 M glycine pH 2.5 was used for elution. Figure 8 shows an exemplary chromatogram of these first attempts (obtained in run no. 1).

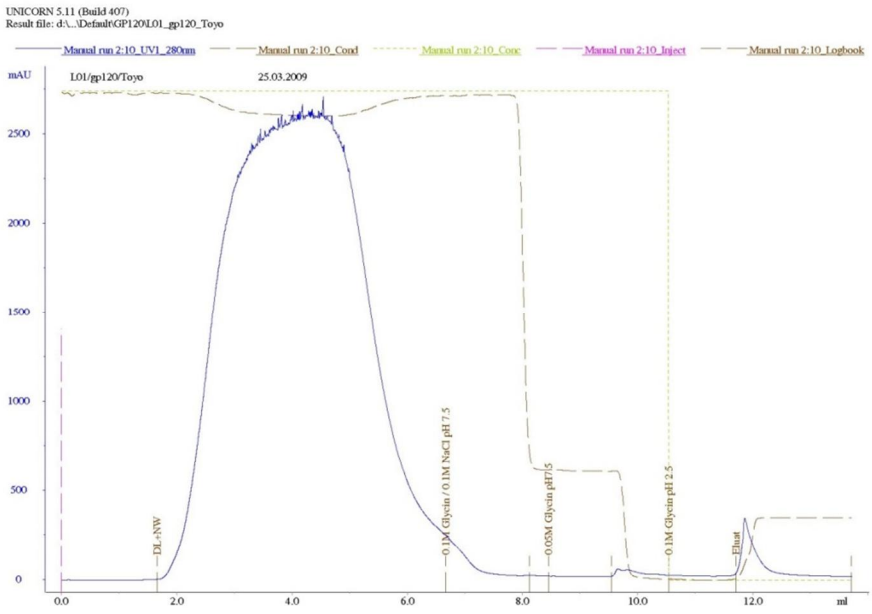


Figure 8: Chromatogram of immunoaffinity chromatography using 2G12 column.

In a first set of experiments, three different columns with varying ligand densities were assessed in one single run per column. Also the first column with low ligand density was used in these experiments.

The individual fractions were analysed on their gp120 content by ELISA and recovery rates were determined. The results of the first runs are given in Table 4 below.

ELISA of runs 1 and 2 was performed using UG37 gp140 as standard. As this protein represents the trimeric form of gp120 including the gp41 domain it is obvious that this test is only semi-quantitative. Thus, a specific standard was required which was prepared out of the eluates of runs 1 - 3. The eluates were further processed by chromatography using a MabSelect column in an established process to remove any antibody eventually leaked from the columns (data not shown). The flow-through was collected. Afterwards it was concentrated and buffer was replaced by 20 mM Tris / 0.5 M NaCl pH 8.3 using Centriprep centrifugational units (Millipore, USA) according to the manufacturer's instructions. Finally, the protein concentration in the resulting retentate was determined by measuring the OD_{280}. Concentration of gp120 was then calculated using a molar extinction coefficient of 72515 (according to ProtParam tool www.expasy.org/cgi-bin/protparam) and determined to be 39.98 µg/ml.

This value was used for preparation of a specific standard. Purity was assessed by SDS-PAGE. The result is shown in Figure 9.

Run No.	1	2	3
Column No.	1	2	3
Column Volume (ml)	1.34	1.35	1.12
Ligand Density (mg/ml)	0.53	2.37	4.77
Applied Volume (ml)	2.9	14	17
gp120 concentration in applied sample (µg/ml)	310.3	310.3	98.0
gp120 applied (µg)	899.9	4344.2	1666.0
gp120 load (µg/ml of resin)	671.6	3217.9	1487.5
Volume DL+NW (ml)	6.86	21.65	26.96
gp120 conc. DL+NW (µg/ml)	31.47	52.35	51.29
total gp120 DL+NW (µg)	215.9	1133.4	1382.8
Volume NW3 (ml)	2.18	3.63	3.89
gp120 conc. NW 3 (µg/ml)	5.10	33.68	1.14
total gp120 NW3 (µg)	11.1	122.3	4.43
Volume Eluate (ml)	2.05	3.02	4.73
gp120 conc. Eluate (µg/ml)	295.63	992.3	24.9
total gp120 Eluate (µg)	606.0	2996.7	117.8
Total Recovery (%)	**92.5**	**97.9**	**90.3**
Yield in Eluate (%)	**67.3**	**69.0**	**7.1**

Table 4: First purification runs using 2G12 affinity columns

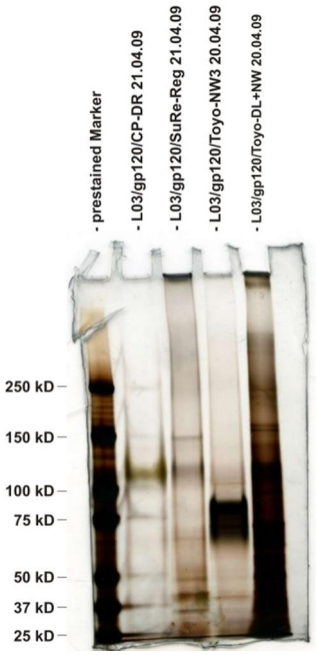


Figure 9: SDS-PAGE of chromatographic fractions. L03/gp120/CP-DR, showing a pronounced band between 100 kD and 150 kD, was used for preparation of an analytical internal standard.

As can be seen, the fraction used for preparation of the standard (L03/gp120/CP-DR) seems to be pure to a sufficient degree. The standard was then prepared by dilution in 1 % BSA in PBS-T to a final concentration of 300 ng/ml. When comparing the gp120 concentration in the applied samples of runs 1 and 2 (310.3 µg/ml) with that of run 3 (98.0 µg/ml) it is obvious that the use of the trimeric gp140 as standard resulted in three-fold higher results.

This emphasizes the importance of the use of the correct reference standard for quantification in ELISAs as different binding characteristics can have a great influence on the results.

All further analyses were performed using the internal IIIB gp120 standard.

Another interesting aspect when comparing the first three purification runs is the low yield in the eluate of run 3. Although the ligand density of column 3 is 2-fold higher than that of column 2, the eluate yield dropped to 7.1 % whereas a yield of 69.0 % was obtained in the eluate of the first run on column 2. This finding complies with experience of gp140 purification where higher ligand densities also resulted in decreased yield (data not shown). This is most likely caused by the high ligand density itself as steric hindrance might prevent gp120 from binding to the antibody or the binding of gp120 to the ligand is weakened due to high ligand density and high amounts of the protein of interest are washed off the column in the first wash step. As the flow-through fractions of loading and the first wash step were collected together, it could not be determined in which way most of the gp120 is lost. However, both types of losses reduce the performance of the column with higher ligand density. As a consequence it was decided to use ligand densities of approximately 2.5 mg/ml of chromatography matrix.

To evaluate the repeatability of the column performance, two additional runs were performed on the column with a ligand density of 2.37 mg/ml.
The following table summarises the results of these purification runs (run no. 4 and 5 using column no. 2).

Run No.	4	5
Column No.	2	2
Column Volume (ml)	1.35	1.35
Ligand density (mg/ml)	2.37	2.37
Applied Volume (ml)	11.2	6.9
gp120 concentration in applied sample (µg/ml)	98.0	111.2
gp120 applied (µg)	1097.6	767.3
gp120 load (µg/ml of resin)	813.0	568.4
Volume DL+NW (ml)	16.27	11.44
gp120 conc. DL+NW (µg/ml)	63.83	52.43
total gp120 DL+NW (µg)	1038.5	599.8
Volume NW3 (ml)	3.71	3.59
gp120 conc. NW 3 (µg/ml)	0.37	0.62
total gp120 NW3 (µg)	1.37	2.23
Volume Eluate (ml)	4.46	3.14
gp120 conc. Eluate (µg/ml)	3.24	14.89
total gp120 Eluate (µg)	14.5	46.75
Total Recovery (%)	**96.1**	**84.6**
Yield in Eluate (%)	**1.3**	**6.1**

Table 5: Results of chromatography runs no. 4 and 5 using column no. 2

Runs 4 and 5 resulted in surprisingly low yields in the respective eluate (1.3 and 6.1 %, respectively) which indicates that performance of the column extensively decreased after the first successful purification run where a yield of 69 % was obtained in the eluate. To investigate if this decrease is caused by the applied elution conditions, the stability of 2G12 as ligand after exposure to low pH was evaluated as described in 3.2.3.1.3. Results are shown in Figure 10.

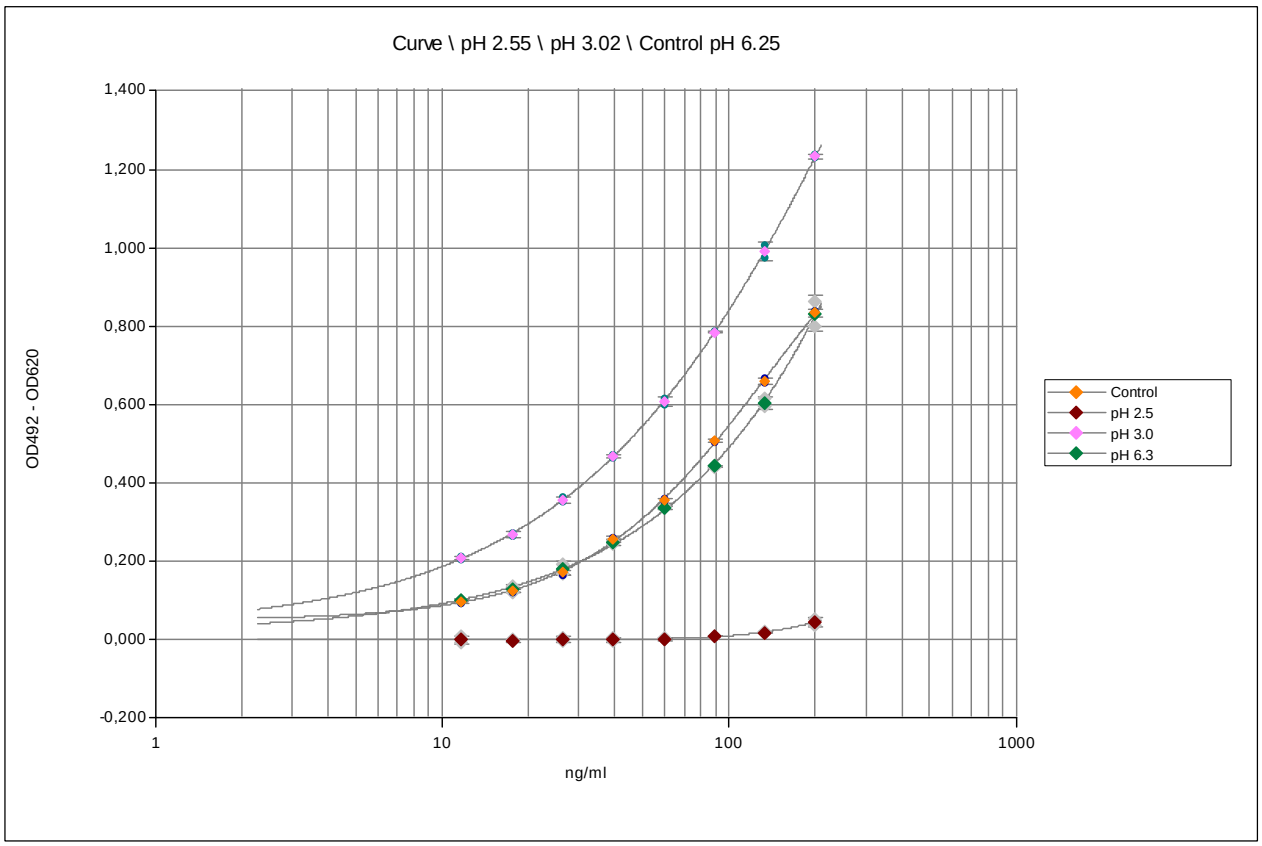


Figure 10: Results of 2G12 stability after exposure to different pH. Untreated 2G12 in dilution buffer (pH 7.2 – 7.4) was used as control.

As can be seen in the figure above the curve 'pH 2.5' is far lower than the others while the pH 3.0 curve is even higher than the standard and the control curve which indicates that the antibody's ability to bind gp120 is lost or at least reduced to an insufficient degree after exposure to a pH of 2.5. pH was reported to have a significant impact on the conformation of an antibody in solution ranging from unfolding to denaturing and aggregation (Kheddo et al., 2014; Thakkar et al., 2012). All of these conformational changes are possible causes for a binding impairment. However, aggregation seems unlikely to occur when the antibody is bound to the chromatography column. Thus, unfolding or denaturing events appear to be the most plausible reasons for the negative effect of low pH treatment on the binding ability of 2G12 antibody to gp120.

As a consequence, different elution buffers were tested:

- 0.1 M glycine, 2 M NaCl, 1.5 M guanidine hydrochloride, pH 7.5
- 0.1 M glycine, 2 M NaCl, 1.5 M guanidine hydrochloride, pH 3.5
- 0.1 M glycine, 2 M NaCl, 1.5 M guanidine hydrochloride, pH 3.0
- 0.1 M glycine, 2 M NaCl, 3 M urea, 2 mM EDTA, pH 7.5
- 0.1 M glycine, 2 M NaCl, 6 M urea, 2 mM EDTA, pH 7.5
- 20 mM citric acid pH 3.0

None of these buffers was found to be suitable for elution as they were not capable to disrupt the binding interaction of gp120 and the antibody (data not shown). Finally, 2 M $MgCl_2$, pH 6.0 was tested. Stability of the antibody after exposure to this buffer was evaluated as described in 3.2.3.1.3. The test was conducted with two independent replicates (rep.1 and rep. 2). Results are shown in Figure 11.

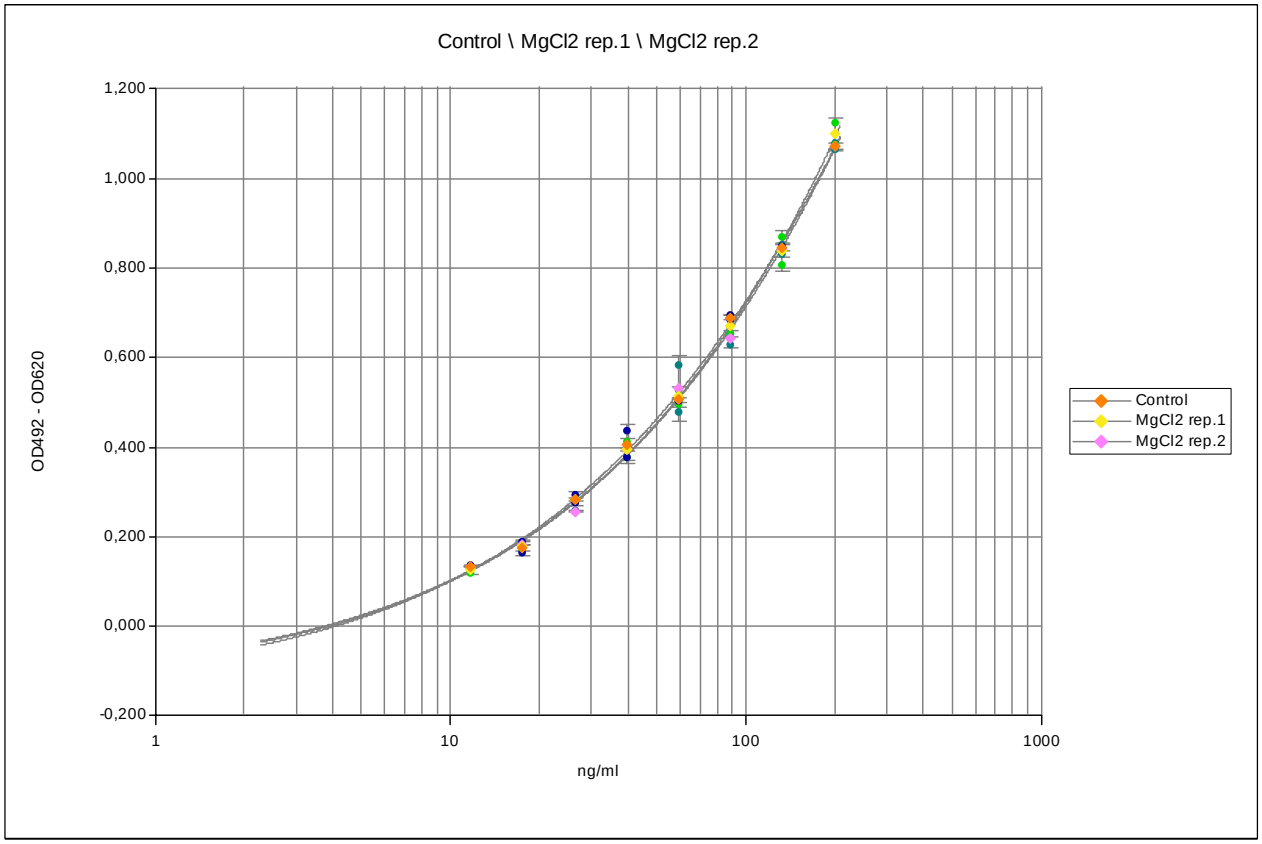


Figure 11: Results of 2G12 stability after exposure to 2 M $MgCl_2$ pH 6.0. Untreated 2G12 in dilution buffer (pH 7.2 – 7.4) was used as control.

The results in the figure above show that the exposure of 2G12 antibody to the chosen elution buffer does not seem to have any denaturing effect on the antibody. Thus, 2 M $MgCl_2$, pH 6.0 was tested in chromatography runs (No. 8 and 9).
Results of these runs using this buffer are shown in Table 6.

Run No.	8	9
Column No.	4	5
Column Volume (ml)	1.26	1.18
Ligand density (mg/ml)	2.12	2.34
Applied Volume (ml)	7.0	7.2
gp120 concentration in applied sample (µg/ml)	98.52	113.5
gp120 applied (µg)	689.6	817.2
gp120 load (µg/ml of resin)	547.3	692.5
Volume DL+NW (ml)	13.22	12.49
gp120 conc. DL+NW (µg/ml)	5.90	0
total gp120 DL+NW (µg)	78.0	0
Volume NW3 (ml)	3.84	4.28
gp120 conc. NW 3 (µg/ml)	3.63	4.77
total gp120 NW3 (µg)	13.94	20.42
Volume Eluate (ml)	3.98	3.93
gp120 conc. Eluate (µg/ml)	160.1	260.6
total gp120 Eluate (µg)	637.2	1024.2
Total Recovery (%)	**105.7**	**127.8**
Yield in Eluate (%)	**92.4**	**125.3**

Table 6: Results of chromatography runs no. 8 and 9 on columns no. 4 and 5.

Results shown in Table 6 above indicate that 2 M $MgCl_2$ is suitable for elution of gp120 bound to a 2G12 column.

In run no. 9, a gp120 yield of 125.3 % was calculated in the eluate. Although recoveries of more than 100 % appear irrational, it has to be kept in mind that every analysis has a certain level of accuracy which is normally evaluated in a validation process. For ligand binding assays (such as ELISA), the European Medicines Agency (EMA) suggests an accuracy of ± 20 % of the nominal value to be met by accuracy samples in the validation in the guideline on biomedical method validation (EMA, 2011). Thus, such a variation can be considered reliable.

In order to enhance buffering capabilities of the elution buffer, glycine was added to the buffer resulting in the elution buffer composed of 2 M $MgCl_2$, 0.05 M glycine and adjusted to pH 6. Results obtained when using this buffer are shown in Table 7.

Run No.	14	17	23
Column No.	5	5	5
Column Volume (ml)	1.18	1.18	1.18
Ligand density (mg/ml)	2.34	2.34	2.34
Applied Volume (ml)	8.61	8.43	13.53
gp120 concentration in applied sample (µg/ml)	50.61	62.93	35.08
gp120 applied (µg)	435.8	530.5	474.6
gp120 load (µg/ml of resin)	369.3	449.6	402.2
Volume DL+NW (ml)	14.98	15.04	18.96
gp120 conc. DL+NW (µg/ml)	8.29	7.17	3.41
total gp120 DL+NW (µg)	124.18	107.84	64.65
Volume NW3 (ml)	4.00	5.19	4.15
gp120 conc. NW 3 (µg/ml)	2.95	2.93	2.24
total gp120 NW3 (µg)	11.80	15.21	9.30
Volume Eluate (ml)	5.10	3.79	3.49
gp120 conc. Eluate (µg/ml)	68.23	118.90	119.88
total gp120 Eluate (µg)	347.97	450.63	418.38
Total Recovery (%)	**111.1**	**108.1**	**103.7**
Yield in Eluate (%)	**79.9**	**84.9**	**88.1**

Table 7: Results of chromatography runs 14, 17 and 23 using column no. 5 and elution buffer containing 0.05 M glycine.

The data given in Table 7 show that the described setup for affinity chromatography is suitable to repeatedly purify gp120 out of clarified cell culture supernatant which was further processed by ultra/diafiltration and achieve satisfying recovery rates in the eluate.

Due to the experience in method development using 2G12 as ligand which resulted in time-consuming experiments for adjusting a suitable elution buffer and to save material, the stability of B12 antibody after exposure to two different elution buffers (0.1 M glycine pH 2.5 and 2 M $MgCl_2$ pH 6.0) was assessed according to 3.2.3.1.4. prior to starting any chromatographic runs. The results are depicted in Figure 12.

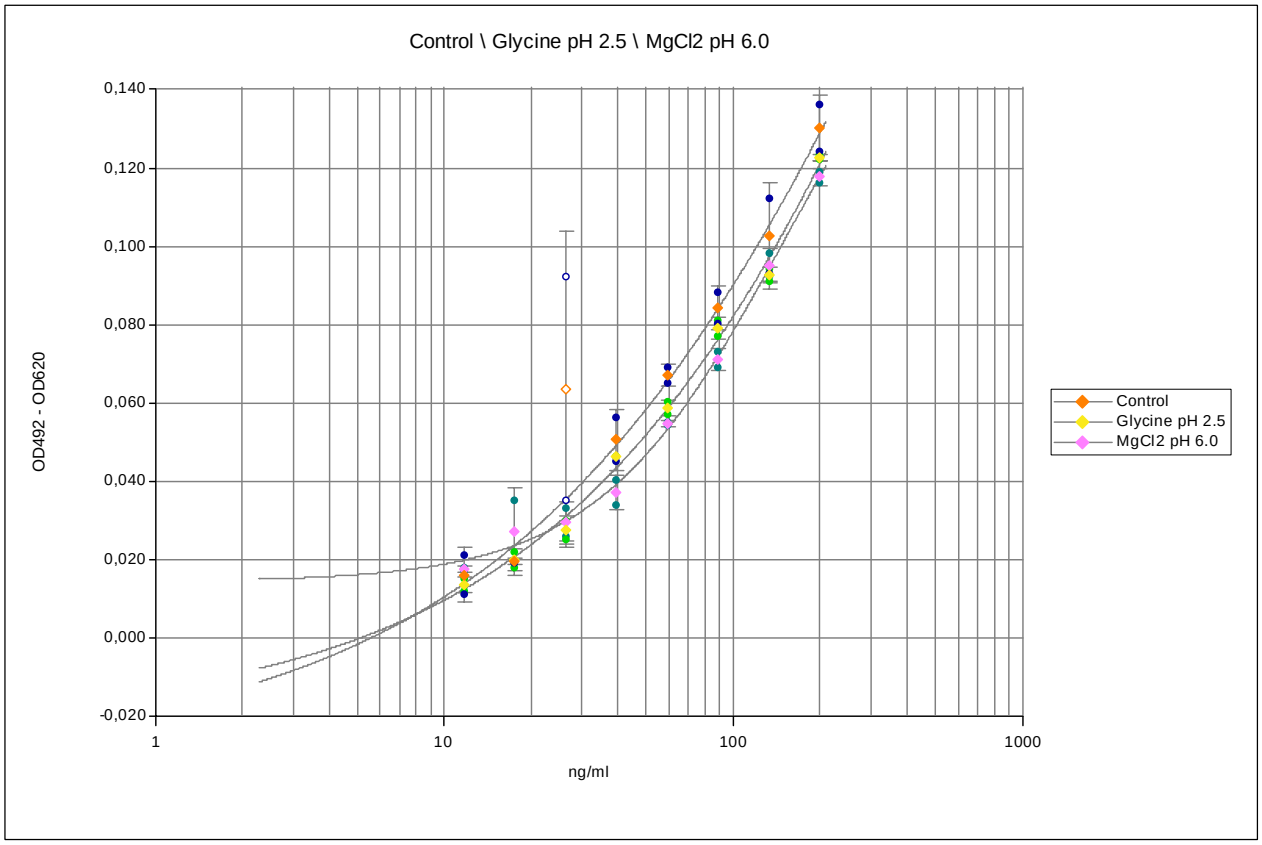


Figure 12: Results of B12 stability after exposure to low pH buffer and $MgCl_2$ buffer. The untreated internal standard in dilution buffer (pH 7.2 – 7.4) was used as control.

In contrast to the results obtained for 2G12, the B12 antibody seems to be more robust and therefore suitable for elution with a low-pH glycine buffer. The similarity of the curves for different buffer conditions and the control are shown accordingly in Figure 12.

Furthermore, the binding stability of the antibody after application of phosphate buffers with different pH was evaluated according to 3.2.3.1.5. Untreated B12 in dilution buffer (pH 7.2 – 7.4) was used as control. The results of this analysis are depicted in Figure 13.

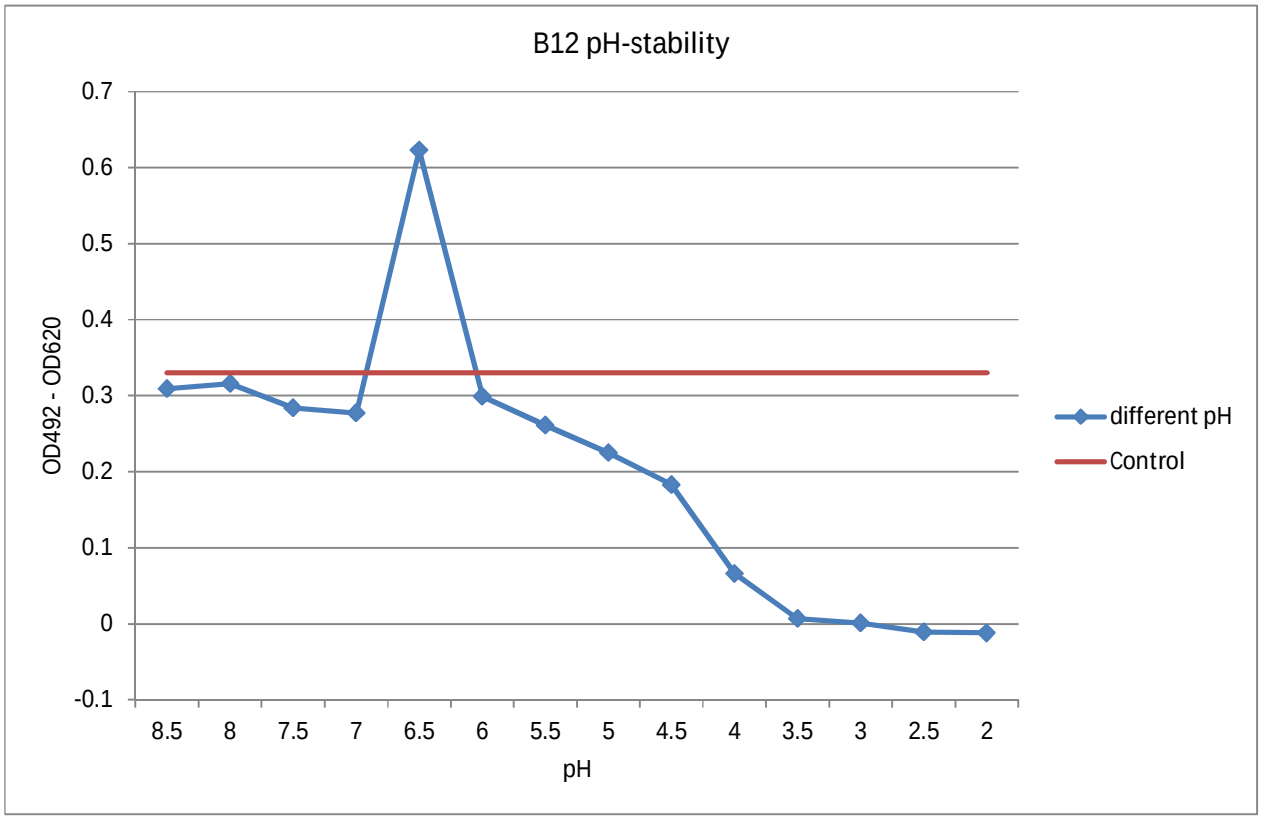


Figure 13: Binding stability of B12 after application of phosphate buffers with different pH.

As can be seen in the figure above, binding can be considered stable from pH 8.5 – 6.0 (with one outlying result at pH 6.5 which was most likely caused by an operator error), decreasing from 5.5 – 3.5 and vanishing below pH 3.0.

As a consequence of these analyses it was decided to use 0.1 M glycine pH 3.0 as elution buffer for affinity chromatography performed on a B12 column as this pH appears to be a good compromise in terms of binding disruption and mild elution condition if concerning that the antibody is still able to bind gp120 after incubation in a buffer with pH 2.5 as demonstrated in the previous experiment.
Three runs were performed on such a column. The results are summarized in Table 8.

Run No.	19	21	22
Column No.	6	6	6
Column Volume (ml)	1.14	1.14	1.14
Ligand density (mg/ml)	2.76	2.76	2.76
Applied Volume (ml)	8.53	9.42	13.38
gp120 concentration in applied sample (µg/ml)	65.27	40.61	35.64
gp120 applied (µg)	556.8	382.6	476.9
gp120 load (µg/ml of resin)	488.4	335.6	418.3
Volume DL+NW (ml)	13.39	14.38	18.95
gp120 conc. DL+NW (µg/ml)	5.38	n/a	4.57
total gp120 DL+NW (µg)	72.04	-	86.60
Volume NW3 (ml)	5.29	3.86	4.28
gp120 conc. NW 3 (µg/ml)	9.23	2.87	4.60
total gp120 NW3 (µg)	48.83	11.08	19.69
Volume Eluate (ml)	2.65	2.58	2.52
gp120 conc. Eluate (µg/ml)	164.20	88.32	166.82
total gp120 Eluate (µg)	435.13	227.87	420.39
Total Recovery (%)	**99.9**	**-**	**110.4**
Yield in Eluate (%)	**78.2**	**59.6**	**88.2**

Table 8: Results of three chromatography runs performed on a B12 column.

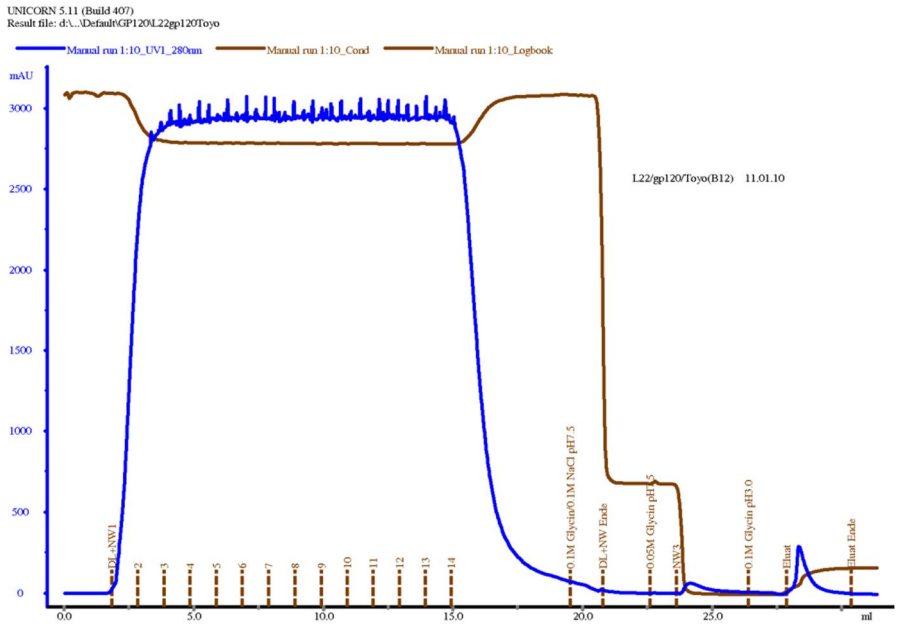
According to the obtained results listed in the table above all three runs can be considered successful. The chosen setups seem to be suitable for purifying gp120 out of clarified cell culture supernatant.

4.3. Comparison of 2G12 and B12 Affinity Chromatography

An important parameter of affinity chromatography is the dynamic binding capacity of the used column which gives indication on how much protein can be purified in one single run. For determination of the dynamic binding capacity a chromatography run was performed on both columns. Sample was applied in an excess and flow-through was collected in aliquots of approximately 1 ml. The content of gp120 in these fractions was determined by ELISA. The breakthrough point was chosen as 15 % of the initial gp120 concentration in the applied

sample. To achieve a high comparability of the two columns, the conditions were chosen as similar as possible for both runs. Thus, the same volumes of sample and wash buffers were applied and flow rates were the same. The chromatograms of both runs are depicted in Figure 14.

14a



14b

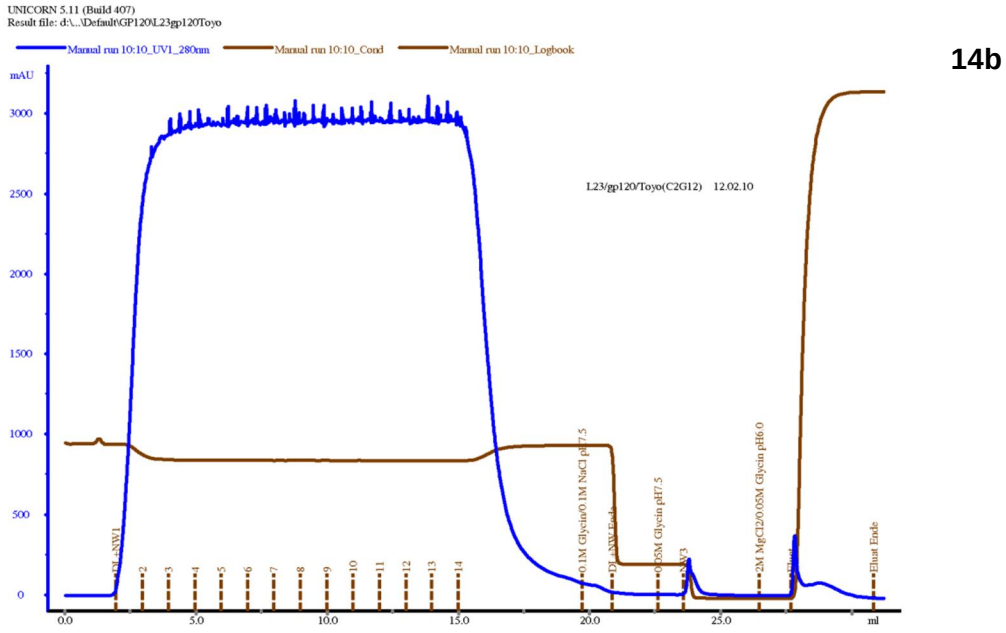


Figure 14: Comparison of B12 (14a) and 2G12 (14b) columns in affinity chromatography. Flow-through was collected in fractions of approximately 1 ml (DL+NW1 – 14) for determination of dynamic binding capacity.

The initial gp120 concentration in the applied samples was determined to be 35.64 µg/ml on the B12 column and 35.08 µg/ml on the 2G12 column. The difference of these two results

when analysing one sample is within the normal variance of an ELISA of up to ± 20 %. For simplification and enhanced comparability the mean value of 35.36 µg/ml was used for calculation of the results depicted in the following figure. The percentage of gp120 concentration in the individual fractions related to the initial concentration was calculated and plotted versus the loaded amount of gp120 normalised on the ligand density to achieve the load in µg per mg of ligand. The breakthrough was chosen to be 15 % which means that a loss of 15 % of the initial gp120 concentration would be tolerated. Furthermore, the 2 curves were compared at a breakthrough level of 10 %. The results are depicted in Figure 15.

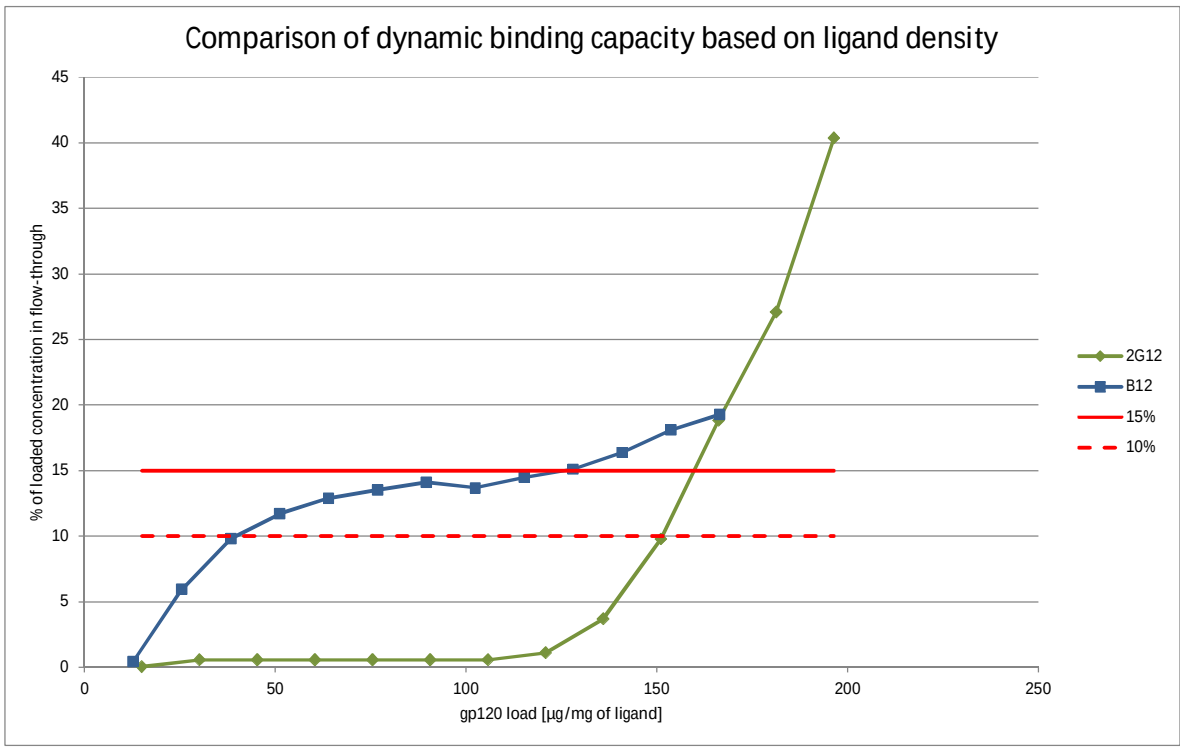


Figure 15: Comparison of the dynamic binding capacity. Gp120 recovery in the individual fractions was determined. Breakthrough was set to 15 % of the initial concentration.

The obtained curves clearly show the difference between the two columns. The curve for the B12 column already shows a significant increase of the gp120 concentration in fractions 2 and 3 and a smooth slope in the following fractions. In contrast, the curve of the 2G12 column remains flat until fraction 8 and shows a strong rise in the consecutive fractions. However, the recovery rates in the respective eluates are similar (88.1 % on the 2G12 column and 88.2 % on the B12 column).

Concerning the dynamic binding capacity, the 2G12 column seems to be the better choice if only the dynamic binding capacity for the 15 %-level ($DBC_{15\%}$) is considered. If the acceptable breakthrough point is defined to a lower limit of 10 % the 2G12 column still shows significantly better performance. Dynamic binding capacities for both columns at the 15 %

and 10 % level are compared in Table 9. On the 15 % - level the 2G12 column's dynamic binding capacity is 25 % higher. On the 10 % - level the dynamic binding capacity of the 2G12 column is nearly 4-fold higher compared to that of the B12 column. Thus, the use of the 2G12 column has to be considered the better choice for this individual application.

Coupled antibody	$DBC_{15\ \%}$	$DBC_{10\ \%}$
2G12	~ 160 $\mu g/mg_{ligand}$	~ 151 $\mu g/ml_{ligand}$
B12	~ 128 $\mu g/ml_{ligand}$	~ 38 $\mu g/ml_{ligand}$

Table 9: Comparison of dynamic binding capacities

In addition, the leakage of the columns was determined. Therefore, the content of human IgG (hu-IgG) in the fractions of two runs was determined as described in 3.2.3.1.7. One of these runs was performed on a 2G12 column (column 5) whereas the other run was performed on the B12 column (column 6). The results are summarized in Table 10.

Coupled antibody	2G12	B12
Run No.	23	19
Column No.	5	6
Hu-IgG content DL+NW [ng]	479.08	910.52
Hu-IgG content NW3 [ng]	3.58	21.16
Hu-IgG content Eluate [ng]	49.65	63.60
Residual hu-IgG Eluate [%]	0.01	0.01
Hu-IgG total (ng)	**532.31**	**995.28**

Table 10: Comparison of column leakage.

The total amount of human IgG found in the fractions of the 2G12 affinity chromatography was 532.31 ng whereas the respective amount in the fractions of the B12 affinity chromatography was approximately 87 % higher (995.28 ng). Unexpectedly, this represents a high difference although it has to be considered that the fractions of the 2G12 column were obtained in the 5th run on the column whereas the fractions of the first run on the B12 column were analysed. It cannot be predicted how the amount of leaching antibody will change over the column lifespan as it depends on several factors like storage duration, operating parameters or used buffers (FDA, 2001). It may be possible that the leakage of the B12 column will decrease in further runs as loosely bound antibody will be washed out over time. However, this assumption cannot be proven with the generated data and additional characterisation runs will be required if a more precise assessment is desired.

Based on the available data the 2G12 column seem to be better concerning the leakage and is therefore to be preferred.

Another important factor which has to be considered during purification of recombinant proteins is the content of residual host cell proteins (HCP). Consequently, the eluates of runs 22 (B12 column) and 23 (2G12 column) were analysed with a commercial ELISA kit as described in 3.2.3.1.6. Results are summarized in Table 11.

Coupled antibody	2G12	B12
Run No.	23	22
Column No.	5	6
HCP concentration [ng/ml]	125.38	28.86
gp120 concentration [µg/ml]	119.88	166.82
Residual HCP [%]	**0.105**	**0.017**

Table 11: Results of HCP determination

Both eluates which were generated from the same initial material only contain marginal amounts of residual HCP (see Table 11). However, with a ratio of only 0.02 % on the B12 column compared to 0.11 % on the 2G12 column, the B12 column shows better performance in this discipline.

However, similar to leaching antibody (see above) re-use and age of column may have an influence on the HCP clearance capacity of the individual columns.

To confirm the identity of gp120 in the eluates from runs 22 and 23 Western Blots were performed using the specific antibodies 2G12 and B12 for detection of the protein on the blot membrane according to 3.2.3.2. Results are shown in Figure 16.

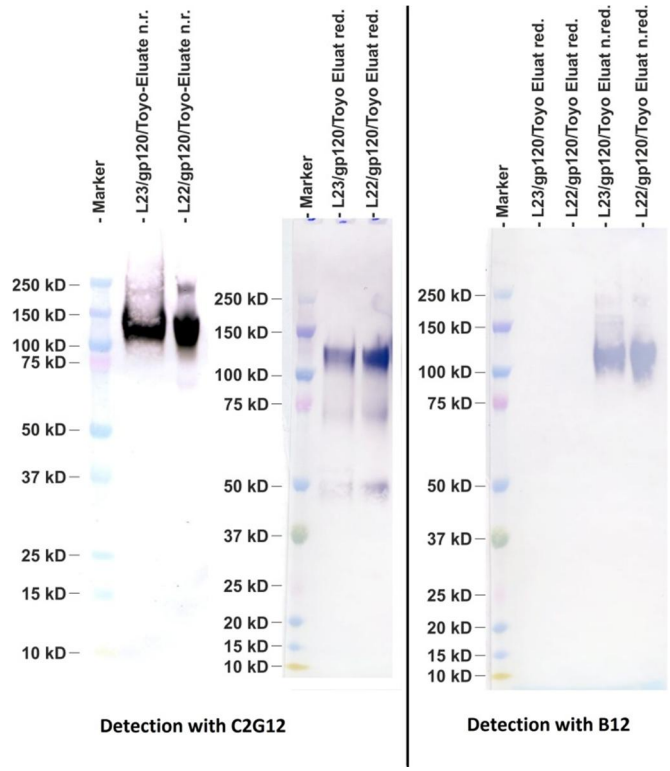


Figure 16: Results of gp120 Western Blots

Both antibodies detect the band at approx. 120 kD as expected when samples are prepared in non-reducing conditions (labelled with n.red. or n.r.). However, the reduced samples (labelled with red.) are not uniformly detected by the different antibodies. While 2G12 detects the band at 120 kD and additional bands at approx. 75 kD and 50 kD, B12 does not detect any band at all. These findings indicate that reducing conditions lead to partial cleavage of gp120 but also conformational change in uncleaved protein resulting in impairment of the B12 binding and suggesting a conformational epitope. Otherwise at least a faint band at 120 kD would be expected as the same concentrations of gp120 were applied in reduced and non-reduced samples. Differences in the intensity of the Western Blot pattern when using 2G12 and B12 as primary antibodies might be caused by different sensitivities of the assays. Differences in the shape of the bands between run no. 22 and run no. 23 (labelled with L22 and L23, respectively) are most likely caused by the different sample matrices as different elution buffers are used in the chromatographic processes.

5. Summary and Outlook

With an estimated number of 2.5 million new infections in 2011, HIV and the resulting acquired immune deficiency syndrome (AIDS) causing 1.7 million deaths keep being a major worldwide problem (UNAIDS, 2013).

Due to its key role in the viral entering process, the HIV envelope protein gp120 is an interesting target protein for its use as research reagent or even in an eventual vaccine.

To be able to provide sufficient and uniform protein the use of a recombinant cell line for protein production and consecutive purification by a specific downstream process appears most favourable. In this downstream process the application of the strong and specific antigen-antibody binding as used in an immunoaffinity chromatography promises high advantages. Polymun Scientific offered 2 antibodies selective for gp120 in the portfolio in 2009: B12 and 2G12. Both antibodies seemed to be potential candidates for use in affinity chromatography.

In a first part of this work the influence of various sodium butyrate concentrations on the gp120 production process in CHO cells was evaluated (see Figure 6 and Figure 7). In the evaluated range of 0 – 0.4 mM, data showed an optimum at a concentration of 0.4 mM. This concentration was used in batch experiments for generation of culture supernatant used in the following chromatographic processes. However, even higher product yields may be achieved by application of higher sodium butyrate concentrations. Thus, further optimization of the production conditions to obtain more protein of interest in single batch cultivation might be possible.

The coupling of the antibodies to the chosen chromatography matrix (AF-Tresyl-650) was found to be reliable and repeatable with coupling ratios between 74 and 96 % (see Table 3). Experiments with different ligand densities (0.53, 2.37 and 4.77 mg/ml of resin, respectively) performed with 2G12, suggest an optimal ligand density of approximately 2.5 mg/ml (see Table 4).

Suitable elution conditions for both antibodies were required and finally successfully optimized. It could be shown that either 2G12 or B12 can be used in affinity chromatography giving repeatable results regarding the recovery in the eluate fractions which was found to be 60 – 88 % (Table 7 and Table 8).

Flow-through experiments for the evaluation of the dynamic binding capacity for the columns coupled with the different antibodies revealed significant differences in the concentration profile of the fractions. While the gp120 concentration remains below 5 % of the initially applied concentration until a load of approximately 136 $\mu g/mg_{ligand}$ when using the 2G12 column, the concentration in the same load range obtained on the B12 column is above the

selected breakthrough point of 15 %. In general the 2G12 column shows a smoother curve with later increase than the B12 column which displays an early increase followed by flattening of the curve (see Figure 15).

Two process-related impurities were assessed accordingly. The content of residual human IgG (hu-IgG) and the amount of host cell proteins in the eluate fractions. While the hu-IgG content was approximately 53 % lower in the eluate received from the 2G12 column, the HCP content was 6-fold lower in the eluate from the B12 column.

In summary, both antibodies are suitable for use in immunoaffinity chromatography. However, differences were observed when evaluating various process characteristics. The amount of HCPs is low in eluate fractions obtained from the two different columns. Consequently, both eluates can be considered similar regarding residual HCPs as possibly both fractions will need to be further processed to remove residual HCPs in an eventual GMP process. Thus, the difference is not significant here. The same is valid for the human IgG content. An additional process step (e.g. protein-A chromatography) will be necessary for both eluates. Consequently, both eluates can be considered equivalent here. However, the impurity profiles may change over time as the column characteristics might alter over the column lifespan. Thus, further evaluation runs are necessary for more representative results which are also more predictive for future use of such columns in an eventual GMP production.

The most obvious difference was obtained in the dynamic binding capacity, as described above.

Taking all the data into account, the antibody 2G12 appears to be the better choice as ligand for purification of gp120 in immunoaffinity chromatography. However, it must not be disregarded that 2G12 may also bind fragmented or denatured protein as indicated in Western Blot (see Figure 16). If occurrence of such product-related impurities is to be expected, further experiments to prove the conformational nature of the B12 epitope will be of value to provide evidence for suitability of B12 use in such a process.

For the future it will be interesting to enhance and adapt the purification process to increase purity of the protein of interest by addition of further purification steps. In addition, the conditions for the chromatographic process using the B12 column may be further adapted to evaluate if dynamic binding capacity can be increased. Furthermore, a synthetic peptide mimicking the CD4 binding site and called mini-CD4 was described by Martin et al. (2002). This peptide also bears the potential to be a highly selective ligand for use in affinity chromatography. It will be interesting to evaluate suitability of this peptide as first experiments gave promising results.

List of Figures

If not stated different, the figures were generated by the author.

List of Abbreviations

AIDS	Acquired Immune Deficiency Syndrome
ART	Anti-retroviral Therapy
BCIP	5-bromo-4-chloro-3-indolyl phosphate
BSA	Bovine Serum Albumin
CCR5	C-C chemokine receptor type 5
CD4	Cluster of Differentiation 4
CHO	Chinese Hamster Ovary
CXCR4	C-X-C chemokine receptor type 4
ELISA	Enzyme-linked Immunosorbent Assay
GMP	Good Manufacturing Practice
gp	Glycoprotein
GS	Glutamine Synthetase
HCP	Host Cell Protein
HIV	Human Immunodeficiency Virus
HRP	Horseradish Peroxidase
MSX	L-Methionine Sulfoximine
NBT	Nitro-Blue Tetrazolium
OPD	o- Phenylenediamine
PAGE	Polyacrylamide Gel Electrophoresis
pNPP	para-Nitrophenylphosphate
PVP	Polyvinylpyrrolidone
RT	Room Temperature
SDS	Sodium Dodecyl Sulphate

List of Tables

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