UPTEC X 04 030
APR 2004

ISSN 1401-2138

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The effect of viral clearance methods on protein quality

Master's degree project

UPPSALA
UNIVERSITET

Molecular Biotechnology Programme

Uppsala University School of Engineering

UPTEC X 04 030 | **Date of issue 2004-04**

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Title (English)

The effect of viral clearance methods on protein quality

Title (Swedish)

Abstract

Viral clearance methods were investigated and product recovery, costs, time consumption, viral clearance and effect on protein quality were addressed. Two model proteins, 35 and 150 kDa, were used. Two different virus filters were tested at pH above and below Ip and with and without 100 mM NaCl. Product recoveries above 90 % were obtained and no effect on protein quality was detected. The 35 kDa protein was treated with INACTINE and IAA, two virus-inactivating agents, under various conditions regarding time and temperature. Protein quality was assayed using SDS-PAGE, RPC, MALDI-TOF and SEC. When the protein was treated under recommended conditions, no effect on protein quality was detected in these assays. However, further experiments are needed to confirm these results.

Keywords

Viral clearance, virus filtration, INACTINE, IAA

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Project name	Sponsors
Language **English**	Security
ISSN 1401-2138	Classification
Supplementary bibliographical information	Pages **44**

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The effect of viral clearance methods on protein quality

Jessika Allard

Sammanfattning

I läkemedelsindustrin används allt fler biomolekyler. När de tillverkas finns det flera potentiella källor för viruskontamination. För att garantera säkerheten hos en produkt innan den testas på människor ingår olika virusreducerande steg i tillverkningsprocessen. Det här examensarbetet syftade till att samla information om olika virusreducerande metoder och undersöka utbyte, tidsåtgång, kostnad, virusreducerande förmåga och inverkan på proteinkvalitet. Två modellproteiner av olika storlek studerades.

Nanofiltrering, för separation av protein och virus, undersöktes. Salt och högre pH gav högre utbyte över de två olika filter som testades. Utbyten över 90% nåddes, men troligtvis kan utbyten nära 100% nås efter optimering av olika parametrar. Ingen inverkan på proteinkvaliteten upptäcktes. Båda filtersorterna kan avskilja små virus såsom parvovirus (18-24 nm).

Två virusinaktiverande substanser studerades under olika förhållanden gällande tid, temperatur och pH. Substanserna angriper virusets genom vilket medför att replikering förhindras. När de användes under rekommenderade förhållanden sågs ingen påverkan på proteinkvaliteten, men det måste verifieras i ytterligare experiment. Den ena substansen verkade öka nedbrytningshastigheten av proteinet under förhållanden då det var mycket reaktivt. Fördelen med dessa två substanser jämfört med S/D (en blandning av lösningsmedel och detergent) är att de är effektiva mot virus utan hölje likaväl som med hölje.

Examensarbete 20p i Molekylär bioteknikprogrammet

Uppsala universitet april 2004

APPENDICES

ABBREVIATIONS

ICH	International Conference on Harmonisation of Technical Requirements for Registration of Pharmaceuticals for Human Use
LRV	Log_{10} reduction value
DF	Direct flow
TF	Tangential flow
DNA	Deoxyribonucleic acid
RNA	Ribonucleic acid
GPT	Gold particle removability test
PVDF	Polyvinylidene fluoride
S/D	Solvent/Detergent
SPR	Surface plasmon resonance
SDS	Sodium dodecyl sulfate
PAGE	Polyacrylamide gel electrophoresis
MALDI-TOF	Matrix assisted laser desorption ionization-time of flight
SEC	Size exclusion chromatography
RP-HPLC	Reversed phase high performance liquid chromatography
NFP	Normal flow Parvovirus
IAA	2-Iodoacetaldehyde
Ip	Isoelectric point
RU	Response units
FPLC	Fast performance liquid chromatography
WFI	Water for injection
TFA	Trifluoroacetic acid
UV	Ultra violet
PBS	Phosphate Buffered Saline

1 Introduction

1.1 Background viral safety

In the pharmaceutical industry there has been a development towards using biomolecules. There are many biotechnology-derived drugs on the market and more in late-stage clinical trials. In the manufacturing of biopharmaceutical products there are many potential sources of viral contamination and it is important to ensure viral safety of the product before testing in man. Regulatory authorities expect at least two robust orthogonal viral clearing steps in the purification process of all recombinant protein cultivated in human and animal cells[1, 2]. A viral clearance step is considered to be robust if the viral titer reduction is independent of variability in the production parameters. The term orthogonal refers to different principals of viral reduction and two orthogonal steps could be filtration, which removes virus and low pH, which inactivates virus. Clearance efficiency is evaluated as a $\log_{10}$ reduction value (LRV), which is the ratio of the viral concentration per unit volume in the suspension before and after treatment. In general a process must be validated to remove or inactivate three to five orders of magnitude more virus than is estimated to be present in the starting material[3]. However, techniques to inactivate or reduce viruses are often harsh and may affect the product negatively. An ideal viral clearance method is fast and uncomplicated. It sufficiently inactivates or reduces viruses without affecting the target product at all.

1.2 Viruses in viral clearance studies

Viruses are infective particles containing a genome in the form of either DNA or RNA. They are obligatory intracellular parasites, which means that they need a host cell for their replication. The genome is kept in a capsid made out of protein. Enveloped viruses also have a lipid membrane. The size of a virus ranges from 18-300 nm. Viruses can be divided into groups depending on what kind of genome they have, and whether they are enveloped or not. These groups show different resistance to physical and chemical inactivation. In a viral clearance study, the LRV for a process is determined for different kinds of viruses. This is done in a spiking study, where virus is deliberately added (spiked) to the starting material. The types of viruses used are categorized into relevant viruses, specific model viruses and non-specific model viruses. Relevant viruses are viruses known to be present in the starting or raw material or viruses with risk to be introduced into the process. Specific model viruses are viruses closely related to a known virus and can be used when a spiking study with the known virus is not possible. Non-specific model viruses are viruses used in studies to show robustness and the process's virus clearing abilities in general. Several viruses with different physical and chemical properties are used. The types and number of viruses used depend on the phase of the clinical trial[1, 4].

1.3 Virus filtration

Filtration is the separation of particles from fluid by passage of that fluid through a permeable medium. In general, particles are removed by a combination of size exclusion and adsorptive retention. Size exclusion should not be dependent on process- or product-related considerations such as pressure, flux, protein concentration and pH. Adsorptive retention on the other hand depends on all these factors since removal is influenced by membrane surface chemistry and electrokinetic or hydrophobic interactions with the membrane surface. Therefore size exclusion is considered to be more robust and it is the preferred method. The

two major membrane filtration systems are direct-flow (DF) filtration also called normal flow filtration, and cross-flow or tangential-flow (TF) filtration. In the DF mode, the flow is across the surface. In the TF mode, the flow is tangential to the surface with part of the fluid passing through and the rest going back to a reservoir. The fluid then circulates until it has passed through the surface. The fluid that has passed through the surface is called permeate whereas the fluid that flows past the filter tangential to the surface is called retentate. The DF mode is easy and fast with high levels of product recovery. However, DF filters require relatively clean process fluids because particulates can penetrate and clog pores. The TF mode is slower but on the other hand, TF filters can handle higher particulate loads. In the manufacturing process it is important not only to remove virus, but also to get high product recovery. Many viral retention filters are either inherently hydrophilic or are hydrophilized to decrease protein binding and enhance product transmission. Regulatory authorities demand that the user of a virus filter performs a physical test of the filter to ensure that it is intact. Such a test is called an integrity test and can be performed before and after usage. Two types of integrity tests are the non-destructive forward-flow test and the destructive gold particle removability test (GPT). The forward-flow test is performed by wetting and pressurizing a membrane filter to establish a stable and low diffusional flow. The filter is integral if the forward-flow value is lower than a specified value. This test can be performed before and after usage. In the GPT, the filter is challenged with a colloidal gold suspension and the concentration of particulates in the pre- and post challenge material is determined by spectrophotometric methods. This test can only be performed post-use of the filter[5]. In this study, filters with two different kinds of membranes will be used. The Planova filter has a hollow-fiber microporous membrane composed of cuprammonium-regenerated cellulose. This membrane is naturally hydrophilic and slightly negatively charged[6]. The Viresolve NFP (Normal Flow Parvovirus) filter has a composite polyvinylidene fluoride (PVDF) membrane composed of three membranes. The composite structure has a supporting layer and a retentive coating. This membrane is also hydrophilic[7]. The Viresolve filter can only be processed in the DF mode, whereas the Planova filter can be processed both in the DF and TF modes.

1.4 Chemical inactivation

Viruses can be reduced either by removal, as in filtration, or by inactivation. A virus is considered to be inactivated when there has been an irreversible loss of viral infectivity. Methods for inactivation can be physical such as heat and radiation or chemical using solvents, detergents, acids, bases, glutaraldehyde or β-propiolactone. The most common methods for virus inactivation are low pH and solvent/detergent (S/D) since they are of relatively low cost and easy to use. Low pH (3.5-4.0) has been shown to inactivate a number of different viruses in a time ranging from a few minutes up to an hour[8]. A disadvantage of this method is that it may be too harsh for proteins but if it works it is fast, cheap and easy to use. Inactivation with S/D treatment works by dissolving the lipid membrane surrounding all enveloped viruses. This renders the viruses non-infective since they no longer can penetrate the cell. The most frequently used combination of S/D is 1 % tri(n-butyl)phosphate (TNBP) and 1 % Triton X-100[9]. Disadvantages of this method is that it does not affect non-enveloped viruses and there may be loss of product since additional steps of removing S/D must be introduced in the process.

INACTINE™ and Iodoacetaldehyde (under development) are two compounds for chemical virus inactivation from Amersham Biosciences. INACTINE is a low molecular weight nitrogen-containing compound. It is thought to inactivate virus by permanently modifying the viral genome and thereby preventing replication. There are two functional components, a

substituted alkyl chain with positive charges and an aziridine group which alkylates nucleophiles when protonated (figure 1). The free aziridine group is highly reactive, but with the positively charged tail there is selectivity for nucleic acids, probably due to a three-step reaction where the positive charges are attracted to the negatively charged phosphates in the backbone. Due to neutralization of the charges, the aziridine group is protonated and thereby activated. Alkylation of predominantly guanosine (G) follows, which can cause ring opening, base loss and strand breakage. The activity increases with lower pH, but the selectivity between nucleic acid and protein decreases below pH 7. After inactivation, the compound is removed by chromatography[10].

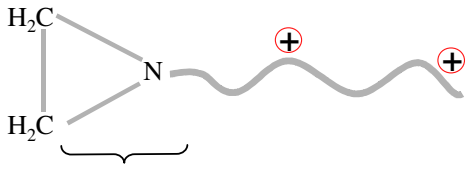

Figure 1. Schematic view of the INACTINE structure

2-Iodoacetaldehyde (IAA) is a new product, not yet on the market. The solid is a dimer hydrate (figure 2), which is converted to monomer and monomer hydrate when dissolved in water. It reacts with the bases adenosine (A), cytosine (C) and guanosine (G) in RNA and DNA. The two-step reaction causes a non-reversible covalent modification of the virus genome, thus preventing correct transcription of the genome for virus replication[11].

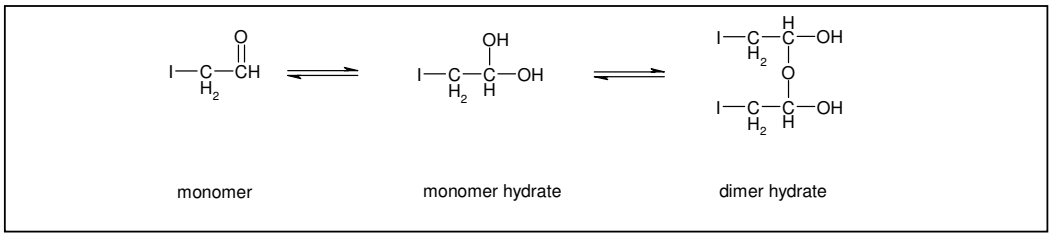

Figure 2. Different forms of Iodoacetaldehyde.

1.5 Model proteins

1.5.1 PROTEIN Z

PROTEIN Z is a code name and will be used throughout this report. The true identity of this protein will not be revealed due to a confidentiality agreement with Biovitrum. PROTEIN Z is a soluble protein present in many body fluids. It has been suggested that its normal function is to regulate lipid degradation. The protein used in this study has been expressed in *E. coli* with a Histidine-tag (His-tag). It is 296 amino acids long with molecular weight MW=34222.1 and isoelectric point Ip=6.01. The main unfolding temperature is
63.2 °C according to differential scanning calorimetry. It is mainly monomeric. There are three domains: α_1, α_2 and α_3 and there is a network of hydrogen bonds between the α_1-α_2 superdomain and the α_3 domain which can explain the high thermal stability. There are four cystein residues, all involved in disulfide bridges[12, 13, 14].

1.5.2 Anti-Apolipoprotein AI (Anti-Apo AI)

Antibodies or antigen-specific immunoglobulines are a part of the immune response. This response is polyclonal and many antibodies with different specificities and affinities for the antigen are produced. Immunoglobulins consist of variable regions responsible for the antigen specificity and constant regions responsible for the basic structure of the protein. In general, an antibody consists of two immunoglobulin (Ig) heavy chains and two light chains. There are different classes of antibodies depending on what kind of heavy chain they have. IgM, IgD, IgG, IgA and IgE are classes in human, mouse and rat. There are also subclasses of IgG and human IgA. There are numerous disulfide bonds, both within each chain and between chains and it is a remarkably stable protein[15]. Anti-Apolipoprotein AI (anti-ApoAI) used in this study is mouse IgG2b with a molecular weight of approximately 150 kDa. It has an isoelectric point (Ip) around 7.

1.6 Methodology

1.6.1 BIACORE

The BIACORE system uses a phenomenon called surface plasmon resonance (SPR) to measure mass attached to a gold/dextran surface. This surface is situated in a flow channel and the dextran layer can be modified to bind protein ligand by amine coupling. On the opposite side of the surface, light is sent in at angles causing total internal reflection. Even if no light passes through the surface, an evanescent electric field reaches out on the other side. At certain combinations of wavelength and angle of the light, this field excites electromagnetic waves called surface plasmons in the metal, resulting in an energy transfer from the reflected light and a decrease in its intensity. If the wavelength is constant, the angle where SPR occurs depends on the refractive index in solution close to the surface. When analyte bind to ligand attached to the dextran layer, there is a change in refractive index. This in turn changes the angle of intensity minimum, which is detected by a two-dimensional diode detector array[16]. The changes are reported in Response Units (RU) and 1000 RU corresponds to a 0.1º change in angle or a 0.001 change in refractive index. This is an approximate change in protein concentration at the surface of 1 ng/mm^2.

1.7 Aim

The aim of this study was to gather information about different virus clearance methods and compare them. Both experimental and literature studies were employed. For each method, clearance efficiency, product recovery, time consumption, price and effect on protein quality were evaluated.

2 *Materials and methods*

2.1 Protein material

PROTEIN Z (1.5 mg/ml and 2.0 mg/ml in PBS buffer) was supplied by Biovitrum. Anti-Apo AI (2.8 mg/ml) was supplied by Mabtech. Apolipoprotein AI (1 mg/ml) was supplied by Academy bio-medical company.

2.2 Virus filtration

The protein (PROTEIN Z or Anti-Apo AI) was diluted in 20 mM Phosphate buffer or Citrate-Phosphate buffer. The virus filters were connected to a Unicorn 3.0-controlled Amersham Biosciences ÄKTA-FPLC system (Inv No 60027274). The on-line UV and conductivity detectors were bypassed in order to lower the backpressure and an analogue pressure gauge was installed preceding the filter in the flow path. Filtration of protein was performed in the DF mode at constant flow.

2.2.1 Planova® filters

Planova filters can be operated in the DF mode as well as in the TF mode. When the filters are washed and equilibrated, fluid is passed both tangentially over the surface as in the TF mode (retentate) and across the surface as in the DF mode (permeate) (figure 3). Planova®15N (Prod No 10301200060) and Planova®20N (Prod No 40305270078) (filter area 0.001 m^2) from Asahi Kasei Corporation were pre-use flushed with WFI (in both retentate and permeate mode). They were equilibrated with 20 ml buffer in retentate mode at constant flow 2 ml/min and 20 ml in permeate mode at constant flow 0.5 ml/min (Planova®15N) or 1.0 ml/min (Planova®20 N). Protein was filtrated and the filters were rinsed with 10 ml buffer at the same constant flow in permeate mode. During filtration, the pressure was manually monitored. Fractions containing protein were identified by off-line absorbance measurements at 280 nm. These fractions were pooled and samples were collected. Load samples were collected before filtration. Post-use the filters were flushed with 0.25 M NaOH, 1 % (w/v) SDS (retentate mode 12 ml at 4.0 ml/min and permeate mode 6 ml at 0.5 ml/min), 0.33 mM HCl (retentate mode 12 ml at 4.0 ml/min and permeate mode 6 ml at 0.5 ml/min) and WFI (retentate mode 12 ml at 4.0 ml/min and permeate mode 6 ml at 0.5 ml/min). After cleaning, the filters were reused. If the filters were not to be used for a few days they were stored in 2 M NaCl at 2-8°C.

Figure 3. Flow paths of the Planova filter. Picture from Planova homepage (ref. 6).

2.2.2 Viresolve® NFP filters

Optiscale™-25 Capsule filter with Viresolve® NFP membrane (filter area 3.5 cm^2) from Millipore (Lot No C3EN60442) were pre-use flushed with approximately 40 ml WFI at 2.0 ml/min and equilibrated with 20 ml buffer. Protein was filtrated and the filter was rinsed with 10 ml buffer at constant flow 2.0 ml/min. During filtration, the pressure was manually monitored. Fractions containing protein were identified by off-line absorbance measurements at 280 nm. These fractions were pooled and samples were collected. Load samples were collected before filtration. After usage, the filters were disposed of.

2.3 Virus inactivation

2.3.1 INACTINE™

INACTINE™ (Lot No 00101) was supplied by Amersham Biosciences. For worst case studies, a 2% stock solution was prepared in 120 mM Citrate buffer, pH 2.5. The stock solution was used immediately after preparation. The protein was diluted in 20 mM Citrate buffer, pH 6.0. The final concentrations were 250 µg/ml PROTEIN Z and 0.2% INACTINE™ and the pH was 6.0. 250 mM Citrate buffer, pH 6.0 was added to the negative control instead of INACTINE™. The samples were incubated at 37˚C in a water bath for 24 hours. For further inactivation studies the 2% stock solution was prepared in 250 mM Phosphate buffer, pH 4.2. PROTEIN Z was diluted in 20 mM Phosphate buffer pH 7.2. The final concentrations were 250 µg/ml PROTEIN Z and 0.1% INACTINE™ and the pH was 7.2. The samples were incubated at different combinations of time and temperature. After inactivation, INACTINE™ was removed or quenched. Removal was achieved by size exclusion chromatography on disposable PD-10 columns packed with Sephadex™ G-25 medium (Amersham Biosciences). Quenching was achieved by adding 125-250 µl quenching solution (25 mM Phosphate buffer, 500 mM Sodiumthiosulphate, pH 7.0) to a 1 ml sample and incubating for two hours at room temperature.

2.3.2 IAA

2-Iodoacetaldehyde (IAA) (Lot No 16075-1) was supplied by Amersham Biosciences. A 50 mM stock solution was prepared in WFI and stored at 2-8˚C. For inactivation studies, samples were prepared with 250 µg/ml PROTEIN Z in 50 mM sodium acetate, pH 5.0 and 5 mM IAA. The samples were incubated at different combinations of time and temperature. Negative controls were treated the same way but without IAA. After inactivation, IAA was removed or quenched. Removal was achieved by size exclusion chromatography on disposable PD-10 columns packed with Sephadex™ G-25 medium (Amersham Biosciences). Quenching was achieved by adding 100 µl quenching solution (25 mM Phosphate buffer, 500 mM Sodiumthiosulphate, pH 7.0) to a 1 ml sample and incubating for two hours at room temperature.

2.4 Analysis

2.4.1 BIACORE

A Biacontrol software-controlled BIACORE Upgrade instrument (Inv No 110455) and BIACORE CM5 Sensorchip of certified grade (Lot No 1121933) were used for concentration determination/binding study. Apolipoprotein AI was diluted in 10 mM sodium-acetate, pH 4.0 to a concentration of 100 µg/ml. 70 µl were injected for immobilization with amine coupling, performed according to BIACORE recommendations. For concentration determinations 35 µl Anti-Apo AI samples were injected and the surface was regenerated using 5 µl Glycine-HCl (equal amounts of pH 1.5 and 3.0). Standard curves were obtained using 10 samples of known concentration ranging from 0.020-10 µg/ml of Anti-Apo AI. Samples of unknown concentration were run in duplicates with three different concentrations (300, 600 and 1200 times diluted). For concentration determination of PROTEIN Z, another surface was prepared in the same way as for Anti-Apo AI. Anti-PROTEIN Z was diluted in 10 mM sodium-acetate, pH 5.5 to a concentration of 20 µg/ml for immobilization. Standard curves were obtained

using 10 samples of known concentration ranging from 0.012-6.0 µg/ml of PROTEIN Z. Samples of unknown concentration were run in duplicates with three different concentrations (300, 600 and 1200 times diluted). All samples were diluted in HBS-P running buffer supplied by BIACORE.

2.4.2 RP-HPLC

A ChemStation software-controlled Agilent 1100 HPLC instrument (Inv No 61000013) was used for the reversed phase-high performance liquid chromatography (RP- HPLC) runs. The column was a Jupiter 5u C18-column, 250×4.6 mm and 5 micron from Phenomenex. Mobile phases were: (A) 0.1% Trifluoroacetic acid (TFA) and (B) Acetonitrile (ACN), 0.1% TFA. The protein was immobilized and eluted using the gradient in figure 4 at constant flow 1.0 ml/min. The protein was detected by on-line absorbance measurements at 220 nm.

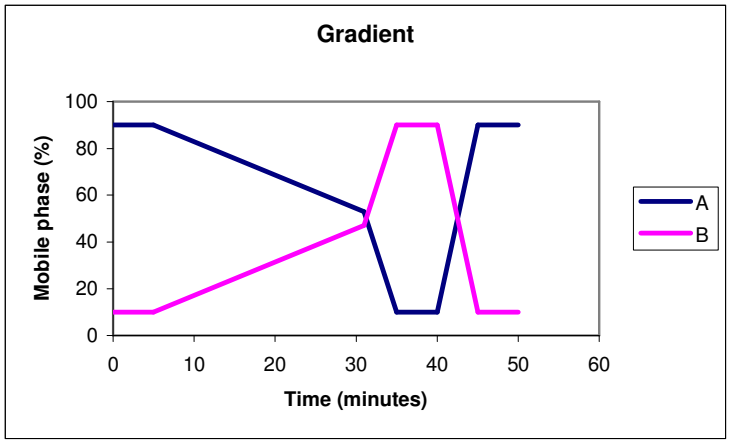

Figure 4. The gradient used in the RP-HPLC analysis.

2.4.3 MALDI-TOF

Matrix assisted laser desorption ionization-time of flight (MALDI-TOF) was used to measure the mass of the protein in order to determine if it had been affected by any of the virus inactivators. 10 mg of the matrix, sinapinic acid, were dissolved in 1000 µl 50 % (v/v) Acetonitrile, 0.05% (v/v) TFA. 1 µl matrix and 1 µl sample were dried in on the sample plate and analyzed in an Applied Biosystems Voyager System 4123 instrument operating in the linear mode.

2.4.4 SEC

Size exclusion chromatography (SEC) was used to monitor separation of protein and virus inactivator substance. 90-100 µl samples were loaded onto a Superdex 75 HR 10/30 column connected to a ChemStation software-controlled Agilent 1100 HPLC instrument (Inv No 61000013). Prior to loading, the column was flushed with water and equilibrated with 50 mM Sodium acetate, pH 5.0. When desired, fractions were collected manually. Protein was detected by on-line absorbance measurements at 280 nm.

2.4.5 SDS-PAGE

Sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE) was used to investigate possible fragmentation of protein and protein content in fractions collected during

SEC runs. Novex® Tris-Glycine 8-16% pre-cast gels with Silver Quest silver staining kit (Invitrogen) and NuPAGE® Novex Bis-Tris 10% pre-cast gels (Invitrogen) with Silver Staining Kit, Protein PlusOne™ (Amersham Biosciences) were used.

3 Results and discussion

3.1 Virus filtration

Two different kinds of filters were compared and the influence of pH and salt on protein permeability was tested. A medium sized protein, PROTEIN Z (35 kDa), and a large protein, Anti-Apo AI (150 kDa) served as model proteins. The different pH values were chosen so that experiments were run both above and below Ip of the protein. Further, costs, time consumption and viral clearance for each filter were addressed.

3.1.1 Protein permeability

3.1.1.1 PROTEIN Z

Product recovery for the different virus filters was measured as the ratio of protein concentration in the post- and pre-filtration solutions. The protein concentration was determined by absorbance measurements at 280 nm and by BIACORE analysis (see Appendix 1). The product recovery of PROTEIN Z over the two different filters is presented in table 1 and in figure 5.

Filter	Protein concentration (µg/ml)	Volume (ml)	pH	NaCl (mM)	Flow rate (ml/min)	Pressure (bar)	Product recovery A280	Product recovery BIACORE
Planova 15N	50	15	7.0	-	0.5	0.7	71 % [1)]	90 %
			5.0			0.6	77 % [1)]	72 %
	150	15	7.0	-	0.5	0.7	87 %	92 %
			5.0			0.6	82 %	74 %
			5.0	100	0.5	0.7	92 %	Not determined
Viresolve NFP Optiscale-25	150	15	7.0	-	1.5	2.1-3.0 [2)]	95 %	95 %
			5.0		2.0	1.8-2.9 [2)]	89 %	83 %
		30	7.0	-	2.0	2.1-3.4 [2)]	97 %	Not determined
		15	5.0	100	2.0	1.9-2.5 [2)]	93 %	Not determined

Table 1. Product recovery of PROTEIN Z in filtration experiments. 1) A280 was out of range (<0.1 AU). Results may not be reliable. 2) The pressure rises continuously during sample filtration.

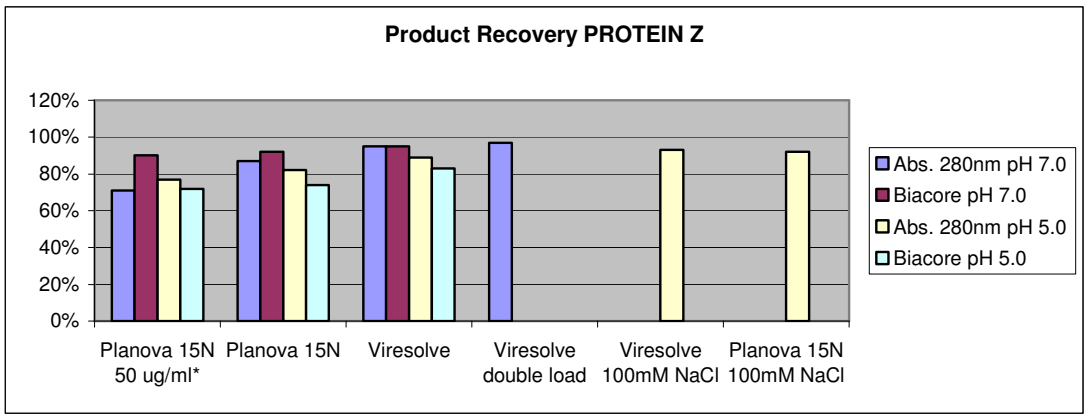

Figure 5. Product recovery from filtration trials with PROTEIN Z. Protein concentration was 150 µg/ml, except for when else is stated, and filtrated volume was 15 ml. *A280 was out of range (<0.1 AU) and results may not be reliable.

The trend was that product recovery increased with higher pH. This may be due to filter membrane properties. The Planova filters have membranes composed of cuprammonium-regenerated cellulose. These membranes are naturally hydrophilic and slightly negatively charged. At the lower pH, the protein is positively charged and an attraction between the filter membrane and the protein may cause a decrease of the permeability. The Viresolve filter is hydrophilic, but whether it is positively or negatively charged is not stated in the manual. Addition of 100 mM NaCl to the protein solution increased product recovery at the lower pH (table 1 and figure 5). This result was consistent with the theory of attraction between filter membrane and protein since increasing the number of ions in the solution would decrease this attraction. The decrease in product recovery could also be due to aggregation of the protein at the lower pH. However, SEC analysis of PROTEIN Z at pH 5.0, performed during the virus inactivation studies, did not show any aggregates (figure 16). Therefore it is not likely that the decrease in product recovery was caused by aggregation. There was no significant difference, at the 0.05 level of the two-sample t-Test (appendix 4), in product recovery between the two filters.

During filtration the pressure was monitored and flow rates were selected to achieve as high flow rates as possible without exceeding the recommended pressure limits. For Planova filters, the maximum pressure is 1 bar and the recommended operating pressure ranges from 0.2-0.8 bar[6]. In this filtration study, the pressure was stable at 0.6 or 0.7 bar (table 1) indicating that there was no clogging of the Planova filter. The maximum pressure for the Viresolve NFP filter is 5.5 bar. Virus spiking studies have been performed at 2.1 and 3.1 bar[7] and thus the flow rate in this filtration study was adjusted to receive a starting pressure of 2 bar. During filtration, the pressure rose with approximately 1 bar in all experiments (table 1) indicating clogging of the filter. This clogging was most likely due to the target protein (PROTEIN Z) as a pure protein solution was used. In an attempt to reach steady state of the pressure, the filtration volume was doubled but there was no stabilization (table 1). This implies that the flow rate selected was too high. In a large-scale process, the flow rate would have to be lowered if the pressure did not reach steady state. Alternatively, filtration could be performed using constant pressure instead of constant flow rate. When 100 mM NaCl was added to a sample, the pressure did not rise as much. This indicates that clogging decreases with addition of salt to the filtration solution.

Considering the pressure, it may seem as though the Planova filter is a better choice than the Viresolve filter. However, the protein load per filter area is much larger for the Viresolve filter than for the Planova filter (table 9). If the protein load could be decreased for the Viresolve filter, the pressure might stabilize at flow rates used in this study.

3.1.1.2 Anti-Apo AI

Product recovery of Anti-Apo AI is presented in table 2 and figure 6. Since Anti-Apo AI is four times as large as PROTEIN Z, the Planova 20N filter with mean pore size 19 nm was chosen, instead of the Planova 15N filter with mean pore size 15 nm, for most of the runs. The Planova 15N filter was tested in one trial.

Filter	Protein concentration (µg/ml)	Volume (ml)	pH	Flow rate (ml/min)	Pressure (bar)	Product recovery A280	Product recovery BIACORE
Planova20N	100	10	8.0	1.0	0.7	94 %	90 %
			6.0		0.7	71 % [1)]	63 % [1)]
	150	10	8.0	1.0	0.7	97 %	94 %
			6.0		0.7	78 %	60 %
Planova15N	150	10	8.0	0.5	0.7	89 %	Not determined
Viresolve NFP Optiscale-25	150	10	8.0	2.0	2.2-3.4 [2)]	92 %	98 %
			6.0		2.0-3.1 [2)]	85 %	89 %

Table 2. Product recovery of Anti-Apo AI in filtration experiments. 1) Two samples absorbing at 280 nm were omitted from the pool. The product recovery is probably somewhat higher than the stated value. 2) The pressure rises continuously during sample filtration.

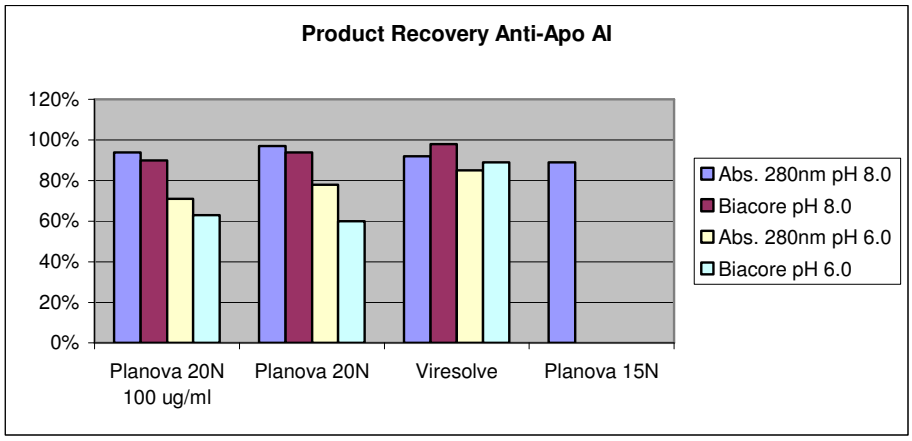

Figure 6. Product recovery from filtration trials using Anti-Apo AI. The protein concentration was 150 µg/ml, unless else is stated, and the filtrated volume was 10 ml.

As with PROTEIN Z, the trend was that product recovery increased with higher pH. However, pH was of less importance for product recovery using the Viresolve filter. The Planova 15N filter was also tested for Anti-Apo AI using the higher pH (table 2 and figure 6). The product recovery was 89 %, which was comparable with product recoveries obtained with the Planova

20N filter for Anti-Apo AI and with the Planova 15N filter for PROTEIN Z. According to the manufacturer, the product recovery is typically greater than 95% with the Planova 15N filter for proteins up to 160 kDa. Anti-Apo AI is approximately 150 kDa and hence it should be possible to achieve good product recoveries for the antibody with the Planova 15N filter. At the higher pH there was no significant difference, at the 0.05 level of the two-sample t-Test (appendix 4), in product recovery between Planova and Viresolve NFP filters. However, the Viresolve filter performed better at the lower pH.

The pressure was stable at 0.7 bar for the Planova filters. For the Viresolve filters, the pressure rose during filtration indicating clogging of the filters (se discussion in section 3.1.1.1).

3.1.1.3 Comments

In the filtration study, Planova filters with filter area 0.001 m^2 were used in order to allow for more run trials. The Asahi Kasei Corporation recommends that filters with at least 0.01 m^2 filter area should be used for product recovery studies to achieve more accurate results. This demands more protein since larger volumes are filtrated through the larger filters. A limited amount of protein was available for this study and therefore the smaller filters were used in order for more run trials. The product recoveries obtained using the 0.001 m^2 Planova filters may differ from the true values because of larger hold-up volumes in relation to filter area and product recoveries obtained in large scale, with the same conditions, would be somewhat higher than small-scale experiments with 0.001 m^2 filters. In this study, the effect of pH and salt on protein permeability was investigated and the conclusion was that higher pH and 100 mM NaCl improved protein permeability. Other parameters that may improve filtration capacity and protein permeability are: decrease in protein concentration and filtration pressure, increase in temperature and/or pre-filtration of the sample[17].

3.1.2 Protein quality

The effect on protein quality was investigated by SDS-PAGE (figure 7). No difference in migration could be observed between non-filtrated and filtrated protein, indicating that the protein stayed intact during the filtration procedures. BIACORE measurements were also used for a quality control. Product recoveries obtained from BIACORE measurements were compared to product recoveries obtained from absorbance measurements (tables 1 and 2). No significant differences, at the 0.05 level of the paired t-Test (appendix 4), could be seen. Since the protein solutions contained only pure PROTEIN Z or Anti-Apo AI, this indicated that there was no change in the binding capacity of filtrated protein compared to non-filtrated. Virus filtration is a relatively mild treatment for proteins. Published data from virus filtration studies on plasma proteins confirm that there is no change in immunogenicity or formation of neoantigens in filtrated plasma proteins[5]. Therefore no effect on protein quality was expected and the results obtained in this study supported this.

3.1.3 Costs and time consumption

Virus filters can only be used once in a process and they are much more expensive as compared with regular filters. Table 8 presents current prices of some filters on the market. Furthermore, the filters must be tested for integrity. Millipore has an air-water diffusion based integrity test for both pre- and post-use test of the system. The test lasts for 15 minutes and assures consistent and reliable virus retention[7]. Asahi Kasei offers a device for automated leakage testing of their filters. This test can also be performed manually. The leak test should be performed pre- and post-use. An additional post-use integrity test should be performed and

Asahi Kasei offers a liquid forward-flow rate (LFR) test and a gold particle removability test for pore size determination[18, 19].

Time consumption is difficult to evaluate. The Viresolve filters operate at much higher flux than the Planova filters, thus reducing the process time. Table 9 presents flux and load values from this study. These values were obtained when operating close to maximum operating pressure for both filters. The Viresolve filter then allows higher flux, but in our tests the pressure was not stable indicating that it might be necessary to operate at lower flux (see discussion in section 3.1.1.1 above). Viresolve filters, but not Planova filters, must be autoclaved before use.

3.1.4 Clearance efficiency

In the virus filter industry, there is no standard for reporting pore size or virus retention capacity. Asahi Kasei filters are rated according to mean pore size. The Planova 15N filter has a mean pore size of 15±2 nm and the Planova 20N filter has a mean pore size of 19±2 nm [6]. However, this does not imply that these filters retain all viruses larger than 15 or 20 nm. The mean pore size gives a rough estimation of protein permeability and virus removal but the viral clearance for the filter needs to be evaluated for each specific process in a scaled down spiking study to get reliable values. Viresolve NFP filters are rated to retain parvoviruses (18-24 nm viruses) with an LRV >4. This means that they should be effective for parvovirus removal, but still the viral clearance needs to be evaluated for each specific process. Clearance efficiency for virus filters has been measured in a number of spiking studies. Table 10 presents LRVs for some representative viruses and bacteriophages for filters obtainable on the market. Bacteriophages are used as models in some virus filtration studies since they are easier to work with. The results give an idea of what sizes of virus the filter retain, but different viruses behave differently and the result is no guarantee that a virus of the same size as the bacteriophage will be retained to the same extension as the phage. The challenge fluid (buffer, protein solution etc) may also affect viral clearance, which is why a viral clearance study needs to be performed under the conditions used in the process with representative load material.

3.2 Virus inactivation

Two virus-inactivating substances, INACTINE and IAA, were tested for their effect on protein quality. The model protein used in this study was PROTEIN Z. Worst case studies and treatment under recommended conditions were performed. Further, viral clearance and time consumption for each substance were addressed.

3.2.1 INACTINE™

PROTEIN Z was first treated with INACTINE under worst-case conditions at low pH, high temperature and high INACTINE concentration for a long time. The purpose was to produce a positive control, with INACTINE bound to the protein, in order to investigate if the analysis methods were sensitive enough to detect it. For comparison a negative control was treated with buffer under the same conditions (table 3).

Sample	INACTINE concentration (%)	Volume (ml)	pH	T (°C)	Time (hours)
A	-	2.5	6.0	37	24
B	0.2	2.5	6.0	37	24

Table 3. The negative control and the worst case conditions for INACTINE treatment of PROTEIN Z.

After removal of INACTINE by size exclusion on PD-10 columns, both samples were analyzed by MALDI-TOF (figure 8) and compared with an untreated sample of PROTEIN Z. Unfortunately the protein is subjected to slow C-terminal degradation (personal communication with Sebastian Bauer, Biovitrum) and the resolution in the MALDI-TOF was not high enough to separate the degradation products. INACTINE bound to the protein would cause an increase of the mass to charge ratio (m/z) by approximately 100, which is comparable with the mass of an amino acid. If every protein molecule had INACTINE attached to it, there would be a shift up of approximately 100 (m/z) in the mass spectrum. This could not be observed, and from the MALDI-TOF data, the conclusion was drawn that, if at all, only a small fraction of protein molecules could have had INACTINE attached to them.

Analysis of the samples using RP-HPLC revealed a small change in the distribution of different forms of the protein for the INACTINE-treated sample compared to the negative control and the untreated sample (figure 9). The concentration of PROTEIN Z was also lower in the INACTINE-treated sample. Fractions were collected and analyzed by MALDI-TOF (figures 10, 11 and 12). Since these fractions contained protein solvated in Acetonitrile and water with 0.1% (v/v) TFA the signal was better than in the first analysis. The mass spectrum for INACTINE-treated PROTEIN Z was rougher with less defined peaks compared to the negative control and the untreated sample, but there was no shift in the mass. This indicated that no INACTINE had bound to the protein.

A series of new trials with INACTINE treatment of PROTEIN Z was performed (table 4, C-G), this time under recommended conditions with pH 7.2 and 0.1% INACTINE. The worst-case trial was also repeated. After removal of INACTINE the samples were analyzed by RP-HPLC. As before, the chromatogram for the worst-case treated sample differed from control- and untreated samples. All other samples had chromatograms, which were close to identical with the chromatogram of the untreated sample (figure 13). This indicated that PROTEIN Z was unaffected when treated under recommended conditions. When treated under worst-case conditions, the results together with MALDI-TOF data indicated that PROTEIN Z was subjected to faster degradation. As there was a difference between worst case treated protein and protein treated under recommended conditions, this strengthened the result that treatment under recommended conditions does not affect protein quality.

Sample	INACTINE concentration (%)	Volume (ml)	pH	T (°C)	Time (hours)	NaCl (mM)
A	-	2.5	6.0	37	24	-
B	0.2	2.5	6.0	37	24	-
C	0.1	2.5	7.2	Room temp.	6	-
D	0.1	2.5	7.2	Room temp.	24	-
E	-	2.5	7.2	Room temp.	24	-
F	0.1	2.5	7.2	37	3	-
G	0.1	2.5	7.2	Room temp.	24	50

Table 4. The different conditions for INACTINE treatment of PROTEIN Z.

To monitor the separation procedure of protein from INACTINE on PD-10 columns, analytical SEC was performed with UV detection of the protein at 280 nm. The trials with INACTINE treatment under worst-case conditions and INACTINE treatment for three hours at 37 ºC were repeated. One part of each sample was treated with sodium thiosulphate, to quench INACTINE activity, and the other part was desalted on a PD-10 column (table 5). Both quenched and PD-10 processed samples were subjected to SEC analysis (figure 14). Chromatograms from the quenched samples contained a peak at 56 minutes, which was not found in the chromatogram from the PD-10 processed sample treated under recommended conditions.

To identify this peak, a sample with untreated PROTEIN Z and a sample with quenching buffer were also analyzed by SEC (figure 16). The results implied that the quenching buffer caused the 56 minutes peak, found in the chromatograms from the quenched samples. In the chromatogram from the PD-10 processed sample of the worst-case treated PROTEIN Z (figure 14) two peaks were found at 55 and 57 minutes. These peaks could be degradation products of PROTEIN Z with bound INACTINE, but this needs to be confirmed in further experiments. Compared to a SEC analysis of standard proteins (data not shown), the size of proteins eluted at these time points should be approximately 3.5 kDa and 2.7 kDa. However, when the samples were analyzed by SDS-PAGE with silver staining to detect protein no bands were seen at this low molecular weight (figure 15). Thus, the peaks were not likely to be due to protein only. Also, the peak at 57 minutes was relatively large compared to the PROTEIN Z-peak, at 38 minutes retention time, and the PROTEIN Z peak was only slightly smaller than the corresponding peak in the PD-10 processed sample treated under recommended conditions (figure 14). If the peaks at 55 and 57 minutes peaks were due to degradation products only, the PROTEIN Z peak would have been smaller. As the chromatogram of the sample treated under recommended conditions only contained the PROTEIN Z peak, this sample was likely to be unaffected by INACTINE.

Sample	INACTINE concentration (%)	Volume (ml)	pH	T (°C)	Time (hours)	PD-10 processed (ml)	Quenched (ml)
B	0.2	3.5	6.0	37	24	2.5	1.0
F	0.1	3.5	7.2	37	3	2.5	1.0

Table 5. Conditions for INACTINE treatment of PROTEIN Z.

3.2.2 IAA

Worst-case conditions for IAA treatment of PROTEIN Z were high temperature and long incubation time. The protein was treated to produce a positive control with IAA bound to it and a negative control to compare with (table 6).

Sample	IAA conc. (mM)	Volume (ml)	pH	T (°C)	Time (hours)	NaCl (mM)
A	-	2.5	5.0	37	24	-
B	5.0	2.5	5.0	37	24	-

Table 6. The worst case conditions for IAA treatment of PROTEIN Z.

After removal of IAA on PD-10 columns, both samples were analyzed by MALDI-TOF (figure 17) and compared with an untreated sample. When IAA binds to its target it looses the iodine atom and left is a 42 Da molecule. In the mass spectrum of the IAA-treated sample there was an increase of 30-40 Da on two sites compared to the negative control and the untreated sample. However, the resolution was not high enough to draw any definite conclusions based on this observation.

The samples were then analyzed using RP-HPLC (figure 18) and fractions were collected. The chromatograms from the treated and untreated samples were very similar in shape but the IAA sample contained less protein than the negative control. Absorbance measurements at 280 nm showed that the concentration of PROTEIN Z in the IAA-treated sample was 68% of the concentration in the negative control. The cause of the protein loss is not clear. However, it was not observed in further experiments with IAA treatment (see below). The RP-HPLC fractions were analyzed by MALDI-TOF (figure 19). The resulting mass spectra were compared to mass spectra from MALDI-TOF analysis of non-treated and control-treated PROTEIN Z (figures 10 and 11), but because the IAA molecule is so small compared to the protein no conclusions could be drawn from this analysis.

A new series of trials was designed with IAA treatment under milder conditions (table 7). The previous worst-case trials were also repeated.

Sample	IAA concentration (mM)	Volume (ml)	pH	T (°C)	Time (hours)	NaCl (mM)	PD-10 processed (ml)	Quenched (ml)
A	-	3.5	5.0	37	24	-	2.5	1.0
B	5.0	3.5	5.0	37	24	-	2.5	1.0
C	5.0	3.5	5.0	Room temp.	6	-	2.5	1.0
D	5.0	3.5	5.0	Room temp.	24	-	2.5	1.0
E	5.0	3.5	5.0	Room temp.	6	100	2.5	1.0
F	5.0	3.5	5.0	37	3	-	2.5	1.0

Table 7. The different conditions for IAA treatment of PROTEIN Z.

One part of each sample was treated with sodium thiosulphate, to quench IAA activity, and the other part was desalted on a PD-10 column. All of the quenched samples and three of the PD-10 processed samples were analyzed by SEC (figures 20, 21 and 22), where the protein was detected by UV at 280 nm. No significant differences in protein concentration between

the IAA-treated samples and the negative control (both quenched and PD-10 processed samples) could be found in this analysis. This was confirmed by off-line absorbance measurements at 280 nm of the PD-10 processed samples (data not shown). In the chromatograms of the quenched samples, three peaks that were not found in the PD-10 processed samples occurred at 56, 69 and 79 minutes retention time (figure 22). The peak at 56 minutes retention time was caused by the quenching buffer (figure 16) and SDS-PAGE with silver staining confirmed that this peak was not due to protein (figure 23). The peaks at 69 and 79 minutes retention time could result from degradation products of IAA, but this needs to be confirmed in further experiments.

3.2.3 Viral clearance and time consumption INACTINE and IAA

The advantage of both INACTINE and IAA over S/D as virus-reducing agents is that they are effective against both enveloped and non-enveloped viruses. There are differences in obtained LRVs of different viruses for the two substances (see Appendix 2 and 3). Both substances clear the small non-enveloped Porcine Parvovirus (PPV) with good LRV. INACTINE is less effective against Hepatitis A virus (HAV) whereas IAA is less effective against Reo-3 (both non-enveloped viruses). The inactivation time using INACTINE is longer compared to S/D (30 minutes to 1 hour) and IAA. In most viral clearance studies an incubation time of 6 hours was used for INACTINE treatment. Inactivation times of 3 hours or less were used in most viral clearance studies with IAA treatment. Most viral clearance studies were performed at room temperature or higher temperatures but many proteins are temperature sensitive and must be kept cold. Both INACTINE and IAA can be used at lower temperatures (4ºC) but the inactivation times are then much longer. INACTINE is compatible with S/D and they can be used simultaneously without compromising the virus reducing ability of INACTINE[10].

4 Conclusions

4.1 Virus filtration

Advantages of virus filtration are ease and the fact that it does not affect protein quality. The mechanism of viral clearance is well understood and removal by size exclusion is a robust viral clearance step. In this study, there was no significant difference between the Viresolve NFP and the Planova filter considering the product recovery. The pressure rose during filtration with the Viresolve filter indicating clogging, but the protein load per filter area was much higher than for the Planova filter. Further experiments using the same load per filter area would be better for comparison of the filters. The Viresolve filter could be used with much higher flux than the Planova filter. This is advantageous since it shortens process time. From this study it was apparent that high pH and 100 mM NaCl are better for protein permeability. However, this study did not result in any final advice as to what filter to use or what process parameters (pH, protein concentration, salt, pressure and flux) to choose since these must all be optimized for each individual protein processed. This study showed that virus filtration works with medium and large proteins (35 kDa and 160 kDa) with reasonable product recovery (over 90 %). Also, the small scale used in this study could have contributed to loss of product because of large hold-up volume compared to filter area. Product recoveries are likely to be better in larger scale. In a manufacturing process, an extra viral clearing step should ideally not cause any loss of product and with some optimization of parameters it may be possible to reach product recoveries close to 100% with virus filtration.

4.2 Virus inactivation

An advantage of virus inactivation using INACTINE and IAA as compared to other chemical inactivation substances (e.g. S/D) is that they are effective against non-enveloped viruses as well as enveloped viruses. The mechanism of viral clearance is understood and several viral clearance studies have been performed. However, none of these agents have been used in a biopharmaceutical purification process and rigorous validation studies would have to be performed in order to secure product quality. The analysis methods used in this study did not detect any influence of either INACTINE or IAA on protein quality when used under recommended conditions. Further experiments would have to be assayed in order to confirm these results. To verify that no compound is bound to PROTEIN Z, a peptide map could be established and comparisons between treated and untreated protein made. A functionality test of the protein would prove that it has not lost any activity. For PROTEIN Z, there is no such test available but for an enzyme, the enzyme activity would be tested. Further investigation of PROTEIN Z could include BIACORE analysis, which would provide information about the binding site to the antibody. If there is no change in binding capacity, this site is unaffected and there is a probability that the whole protein is unaffected.

5 Tables and figures

Company	Filter	Size	Typical processing volume* (liters)	Price (SEK)
Asahi Kasei	Planova 15N	0.12 m^2	-	13500
Millipore	Viresolve NFR	0.08 m^2 (4-inch Opticap)	40	7333
		0.48 m^2 (10-inch Cartridge)	250	12000
	Viresolve NFP	0.08 m^2 (4-inch Opticap)	40	10333
		0.48 m^2 (10-inch Cartridge)	250	34000

Table 8. Current prices of virus filters. *Depending on load material and protein concentration.

Filter	Flow rate (ml/min)	Prot.conc (µg/ml)	Volume (ml)	Flux ($l/m^2/h$)	Load ($µg/cm^2$)
Filtration of PROTEIN Z					
Planova15N	0.5	50	15	30	75
Planova15N	0.5	150	15	30	225
Viresolve NFP Optiscale-25	1.5	150	15	257	643
Viresolve NFP Optiscale-25	2.0	150	15	343	643
Viresolve NFP Optiscale-25	2.0	150	30	343	1286
Filtration of Anti-Apo AI					
Planova20N	1.0	100	10	60	100
Planova20N	1.0	150	10	60	150
Viresolve NFP Optiscale-25	2.0	150	10	343	429
Planova15N	0.5	150	10	30	150

Table 9. Flux and load for the filters in this study.

Filter	(Parvovirus) ssDNA non-enveloped 18-24 nm	Poliovirus (Picornavirus) ssRNA non-enveloped 25-30 nm	SV40 (Polyomavirus) dsDNA non-enveloped 40-50 nm	BVDV (Pestivirus) enveloped ssRNA 50-70 nm	MuLV (Type C Oncovirus) enveloped RNA 80-110 nm	Bacteriophage ΦX-174 28 nm
Planova 15N	>6.2	>6.7	>4.8	≥5.8	-	-
Planova 20N	>4.3	≥8.0	≥5.8	≥7.7	≥6.9	-
Viresolve NFP	>4	-	-	-	-	≥4

Table 10. Log_{10} reduction values (LRVs) for different filters in spiking studies with viruses and bacteriophages. SV40 = Simian virus 40, BVDV= Bovine viral diarrhoea virus, MuLV = Moloney murine leukemia virus. Data were collected from Planova®15N Validation Guide, Version 1.0 issued Mar. 1, 2003, Planova®20N Validation Guide, Version 1.0 issued Jun. 1, 2002, Aranha, H. Viral clearance strategies for biopharmaceutical safety Part 2, *BioPharm*, February 2001 and home pages of the manufacturers.

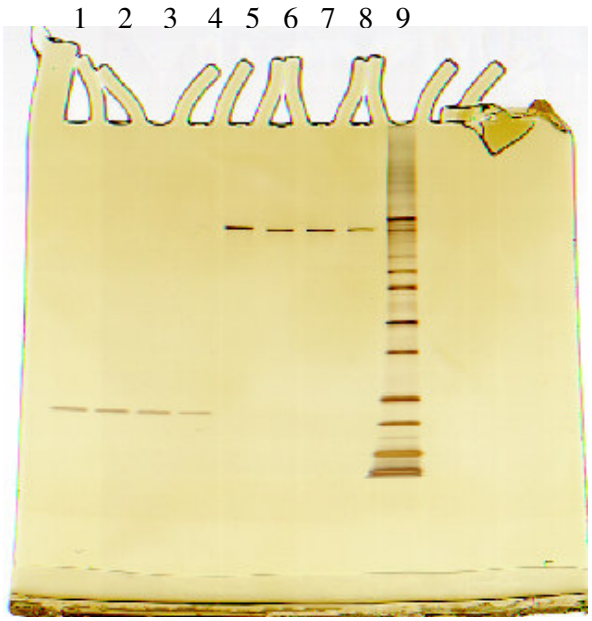

Figure 7. SDS-PAGE with Novex® Tris-Glycine 8-16% pre-cast gel. Silver staining was used for visualization of protein. 1) Non-filtrated PROTEIN Z pH 7.0, 2) Filtrated PROTEIN Z pH 7.0, 3) Non-filtrated PROTEIN Z pH 5.0, 4) Filtrated PROTEIN Z pH 5.0, 5) Non-filtrated Anti-Apo AI pH 6.0, 6) Filtrated Anti-Apo AI pH 6.0, 7) Non-filtrated Anti-Apo AI pH 8.0, 8) Filtrated Anti-Apo AI pH 8.0, 9) Mark-12 marker.

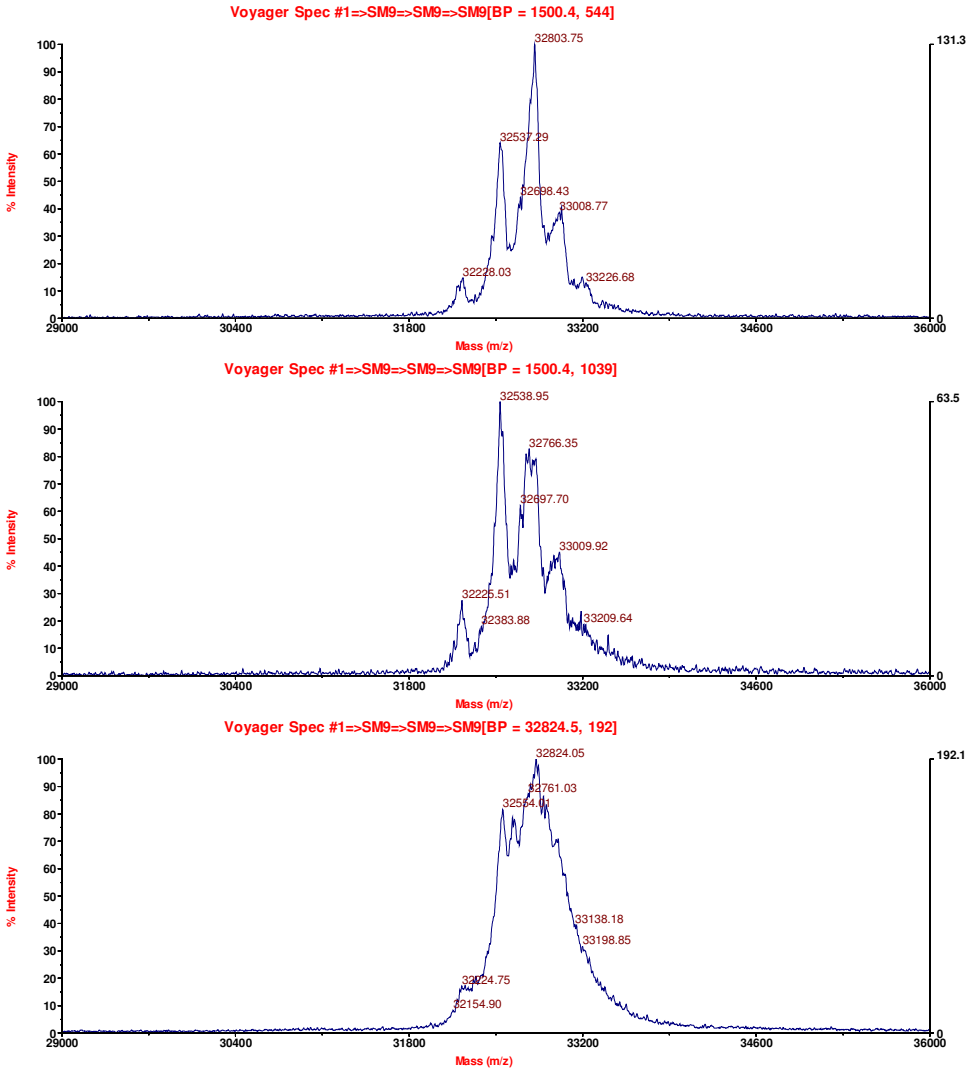

Figure 8. Mass spectra from MALDI-TOF analysis of PROTEIN Z. Top. Non-treated PROTEIN Z, Middle. Control-treated for 24 hours at 37ºC, pH 6.0, Bottom. 0.2% INACTINE for 24 hours at 37ºC, pH 6.0

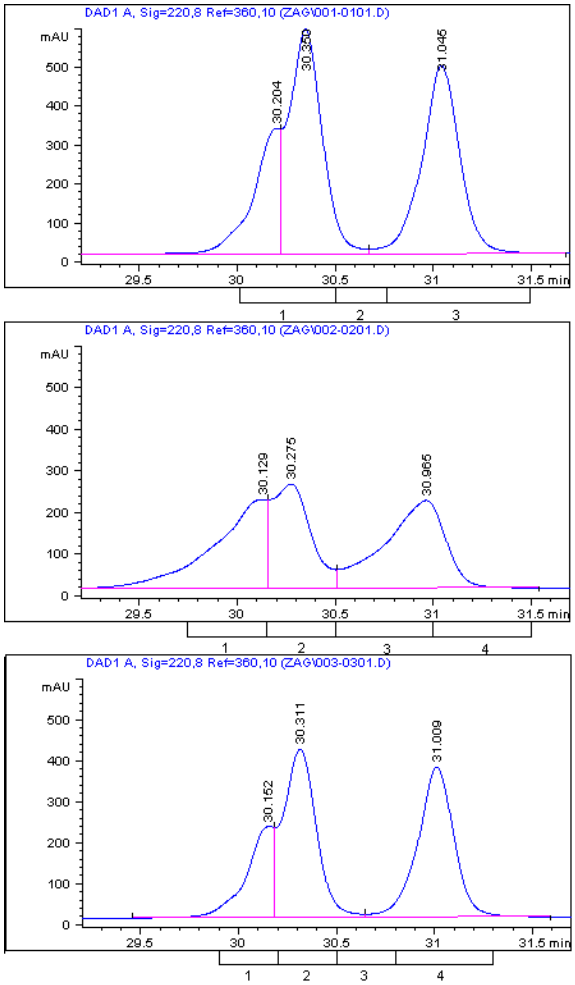

Figure 9. Chromatograms from RP-HPLC runs with PROTEIN Z. Top: Non-treated PROTEIN Z. Middle: PROTEIN Z treated with 0.2% INACTINE for 24 hours at 37 ºC and pH 6.0. Bottom: Control-treated PROTEIN Z, 24 hours at 37 ºC and pH 6.0. Fractions collected are marked under each chromatogram.

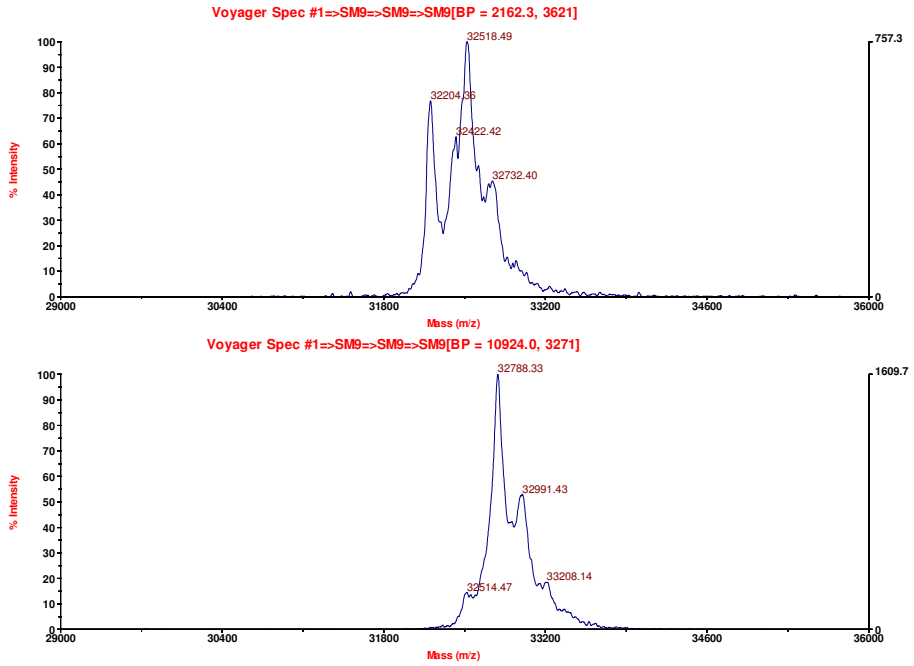

Figure 10. Mass spectra from MALDI-TOF analysis of RP-HPLC fractions of non-treated PROTEIN Z (see figure 9 top). Top. Fraction 1, Bottom. Fraction 3.

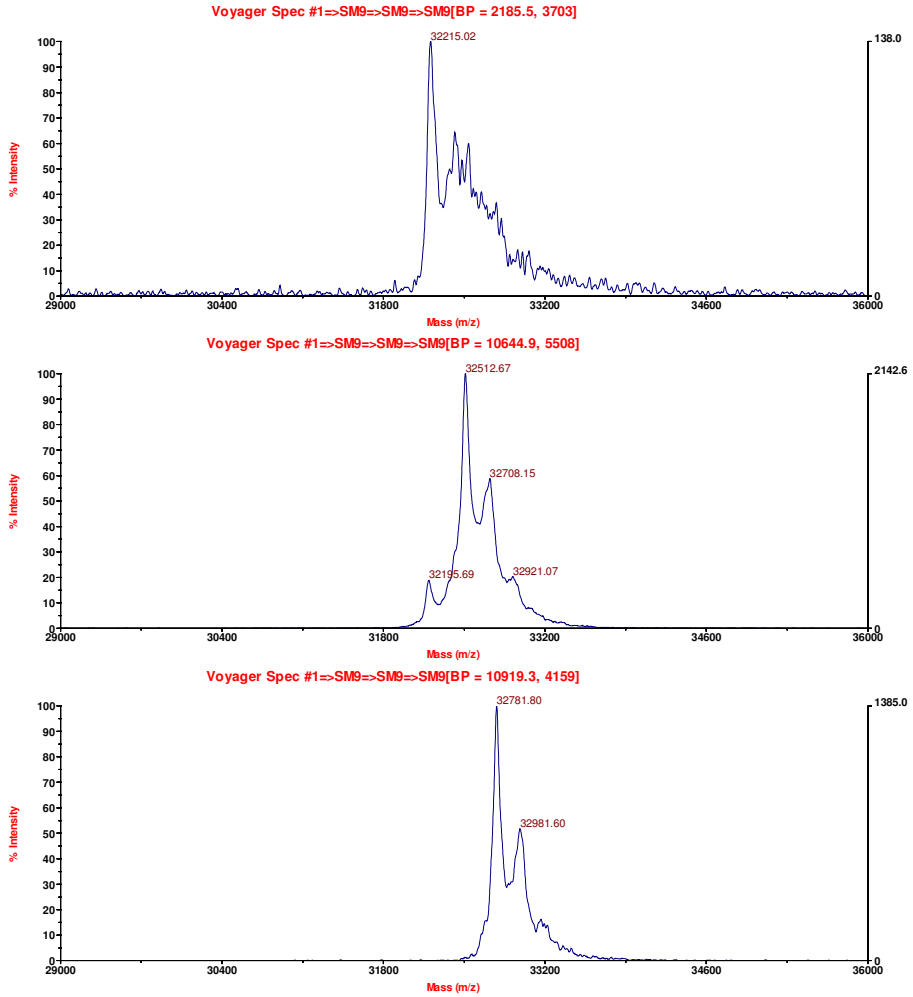

Figure 11. Mass spectra from MALDI-TOF analysis of RP-HPLC fractions of PROTEIN Z control-treated for 24 hours at 37ºC, pH 6.0 (see figure 9 bottom). Top. Fraction 1 (bad signal), Middle. Fraction 2, Bottom. Fraction 4.

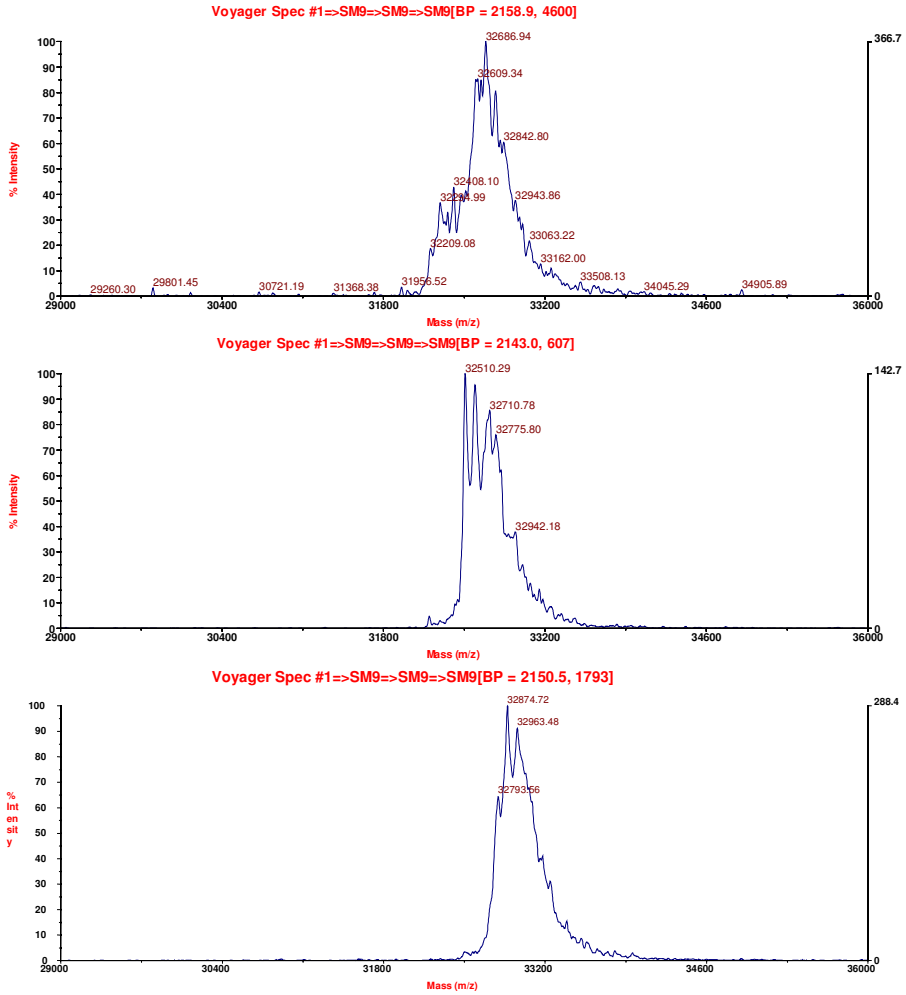

Figure 12. Mass spectra from MALDI-TOF analysis of RP-HPLC fractions of PROTEIN Z treated with 0.2 % INACTINE for 24 hours at 37ºC, pH 6.0 Top (see figure 9 middle). Fraction 1, Middle. Fraction 2, Bottom. Fractions 3-4.

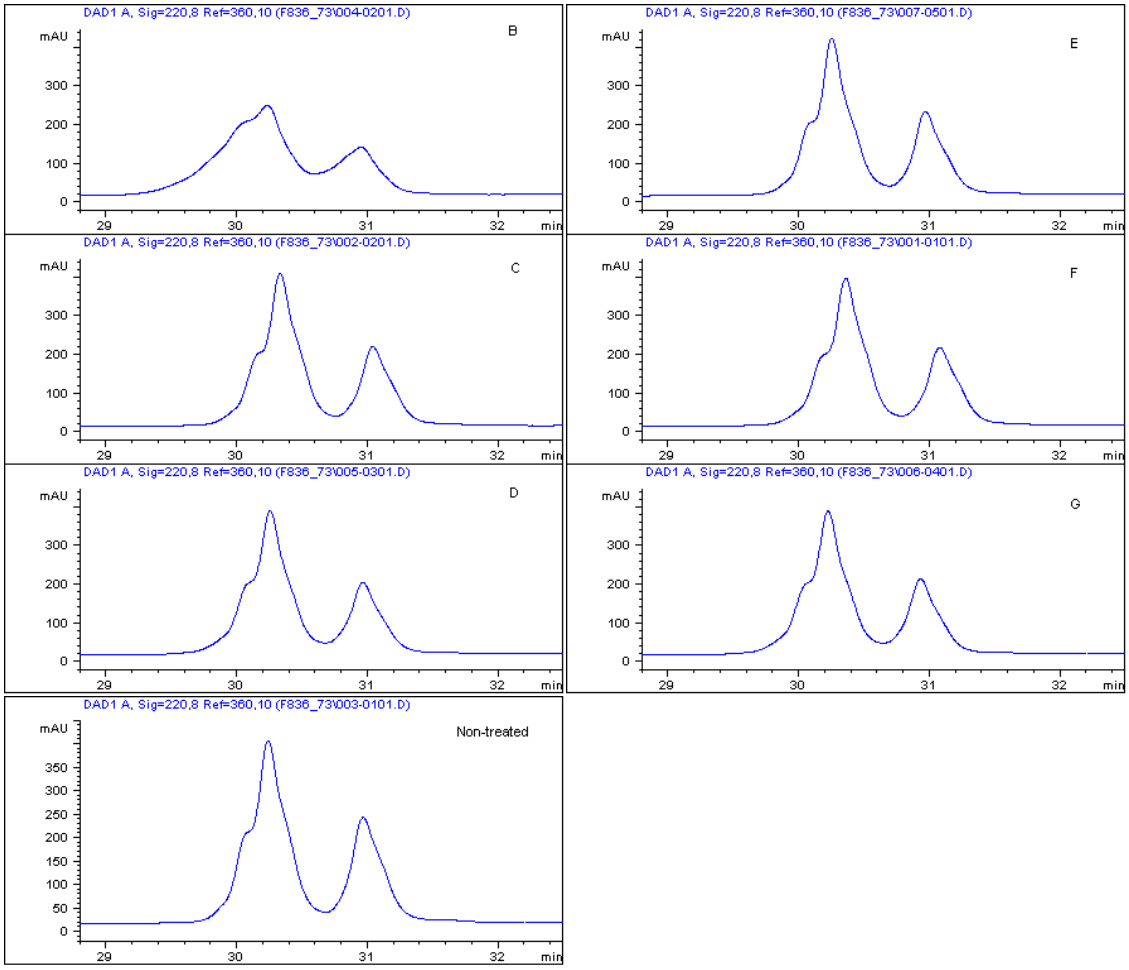

Figure 13. Chromatograms from RP-HPLC runs with PROTEIN Z. Samples B-G were treated according to table 4. B) 0.2% INACTINE for 24 hours at 37 ºC and pH 6.0. Samples C, D, F and G were treated with 0.1 % INACTINE at pH 7.2, C) 24 hours at room temperature, D) 24 hours at 37 ºC, E) Control-treated PROTEIN Z, 24 hours at 37 ºC, F) 3 hours at 37 ºC, G) 24 hours at room temperature, 50 mM NaCl. Non-treated sample was diluted in 50 mM Na-acetate, pH 5.0

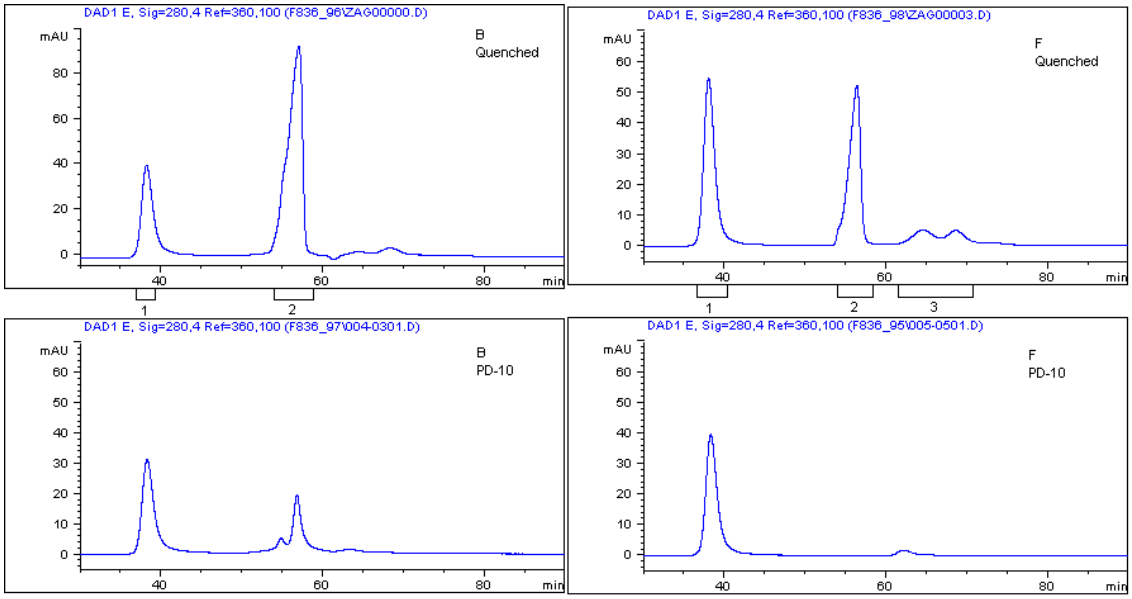

Figure 14. Chromatograms from SEC runs with PROTEIN Z. Samples were treated with INACTINE according to table 6. B) 0.2% INACTINE for 24 hours at 37 ºC, pH 6.0, F) 0.1% INACTINE for 3 hours at 37 ºC, pH 7.2. Fractions, if collected, are marked under respective chromatogram.

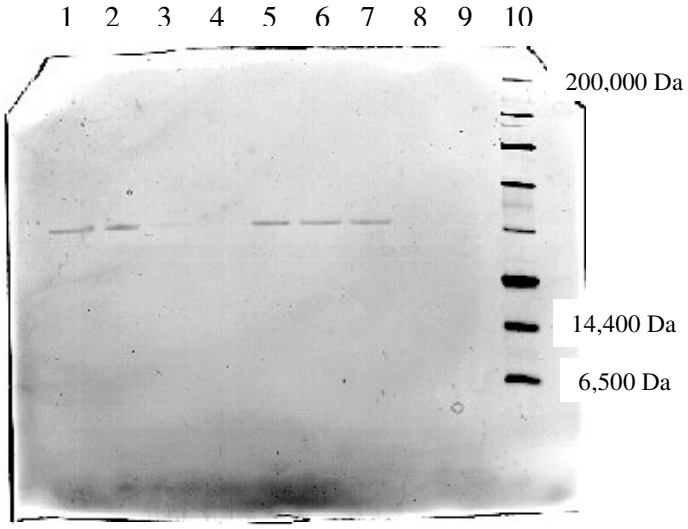

Figure 15. SDS-PAGE with NuPAGE® Novex Bis-Tris 10% pre-cast gel and silver staining. Samples from the left: Positions 1-4. PROTEIN Z treated with 0.2% INACTINE for 24 hours at 37 ºC, pH 6.0. 1) Quenched sample, 2) PD-10 processed sample, 3) and 4) fractions 1 and 2 collected during SEC run (figure 14). Positions 5-9. PROTEIN Z treated with 0.1% INACTINE for 3 hours at 37 ºC, pH 7.2, 5) Quenched sample, 6) PD-10 processed sample, 7)-9) Fractions 1-3 collected during SEC run (figure 14), 10) Broad range marker.

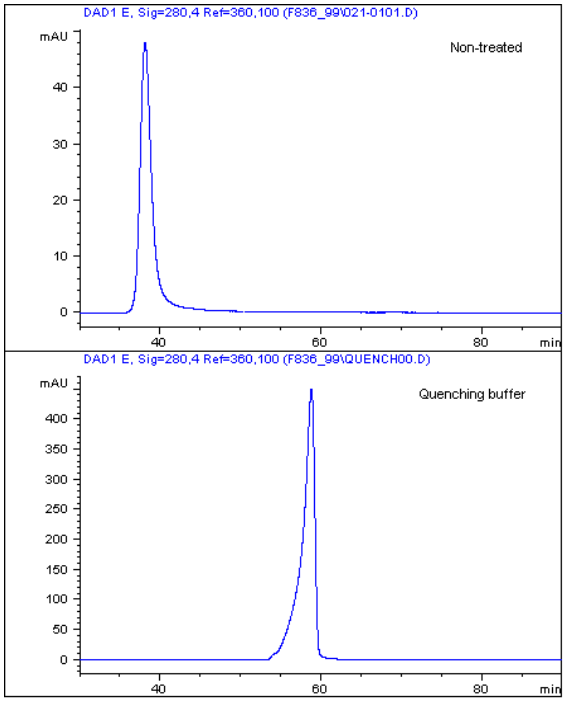

Figure 16. Chromatograms from SEC analysis. Top. Non-treated PROTEIN Z diluted in 50 mM Na-acetate, pH 5.0, Bottom. Quenching buffer (25 mM Phosphate buffer, 500 mM Sodiumthiosulphate, pH 7.0.) The chromatograms are scaled differently.

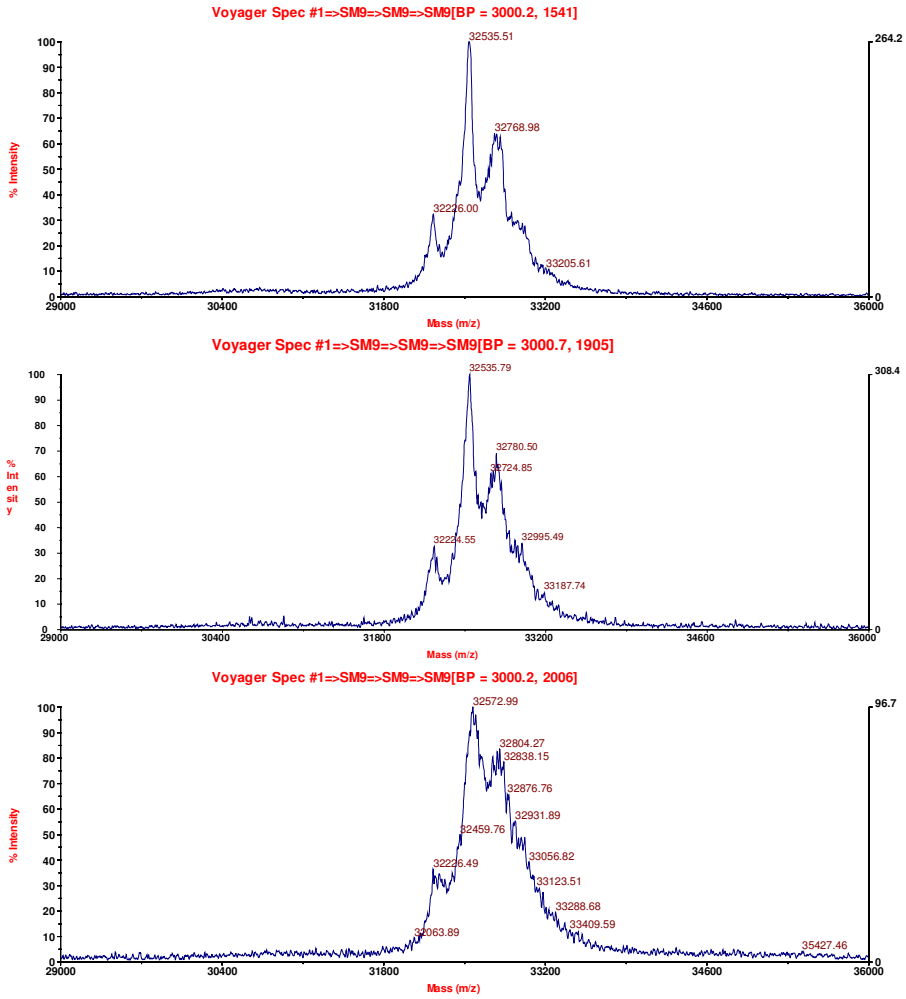

Figure 17. Mass spectra from MALDI-TOF analysis of PROTEIN Z. Top. Non-treated PROTEIN Z, Middle. Control-treated for 24 hours at 37ºC, pH 5.0, Bottom. 5 mM IAA for 24 hours at 37ºC, pH 5.0

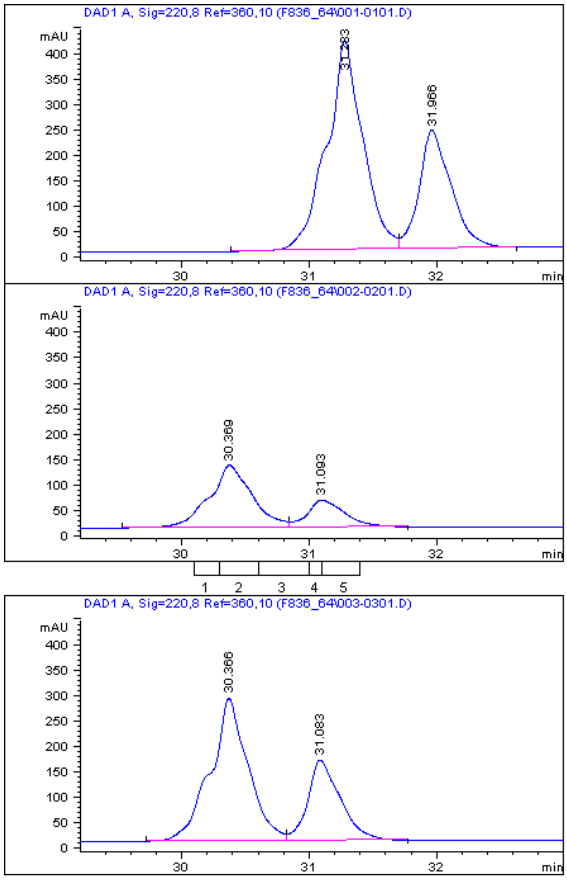

Figure 18. Chromatograms from RP-HPLC run with PROTEIN Z. Top: Non-treated PROTEIN Z. Middle: PROTEIN Z treated with 5 mM IAA for 24 hours at 37 ºC and pH 5.0. Bottom: Control-treated PROTEIN Z, 24 hours at 37 ºC and pH 5.0. Fractions, if collected, are marked under the respective chromatogram.

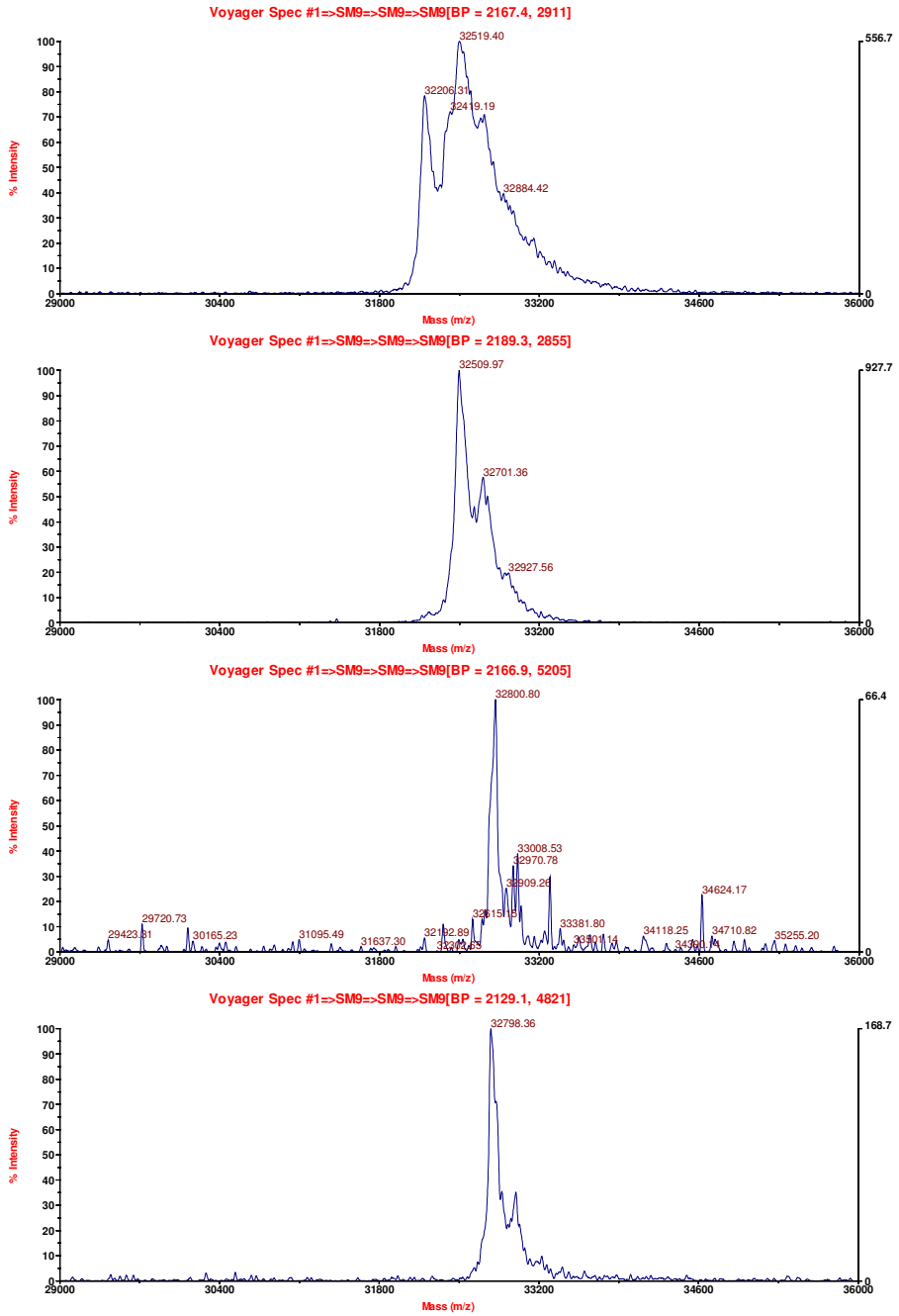

Figure 19. Mass spectra from MALDI-TOF analysis of RP-HPLC fractions of IAA-treated PROTEIN Z (see figure 18 middle). From top to bottom: fraction 1, fraction 2, fraction 4 and fraction 5. The signal was low for fractions four and five.

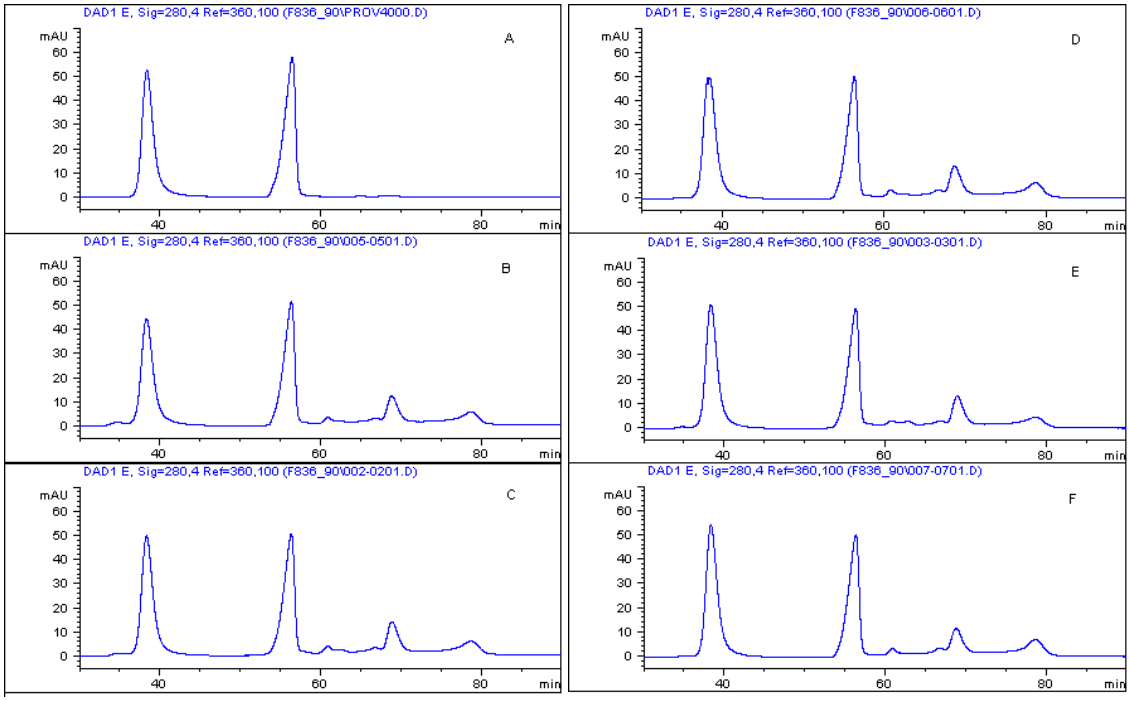

Figure 20. Chromatograms from SEC runs with PROTEIN Z. Samples were treated according to table 7 and quenched with sodium thiosulphate. Sample A. Control-treated PROTEIN Z, 24 hours at 37 ºC. Samples B-F were treated with 5 mM IAA at pH 5.0, B) 24 hours at 37 ºC, C) 6 hours at room temperature, D) 24 hours at room temperature E) 6 hours at room temperature, 100 mM NaCl, F) 3 hours at 37 ºC.

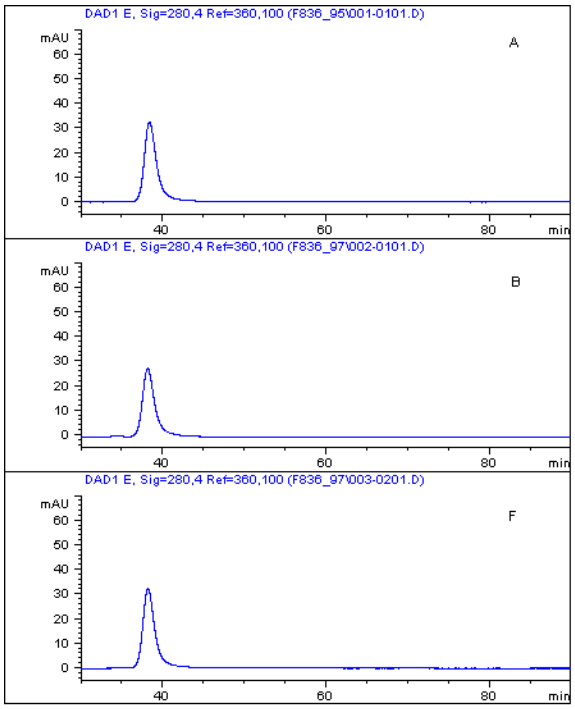

Figure 21. Chromatograms from SEC run with PROTEIN Z. Samples were treated according to table 7 and desalted on PD-10 columns. A) Control-treated PROTEIN Z, 24 hours at 37 ºC. B) and F) were treated with 5 mM IAA at pH 5.0. B) 24 hours at 37 ºC, F) 3 hours at 37 ºC.

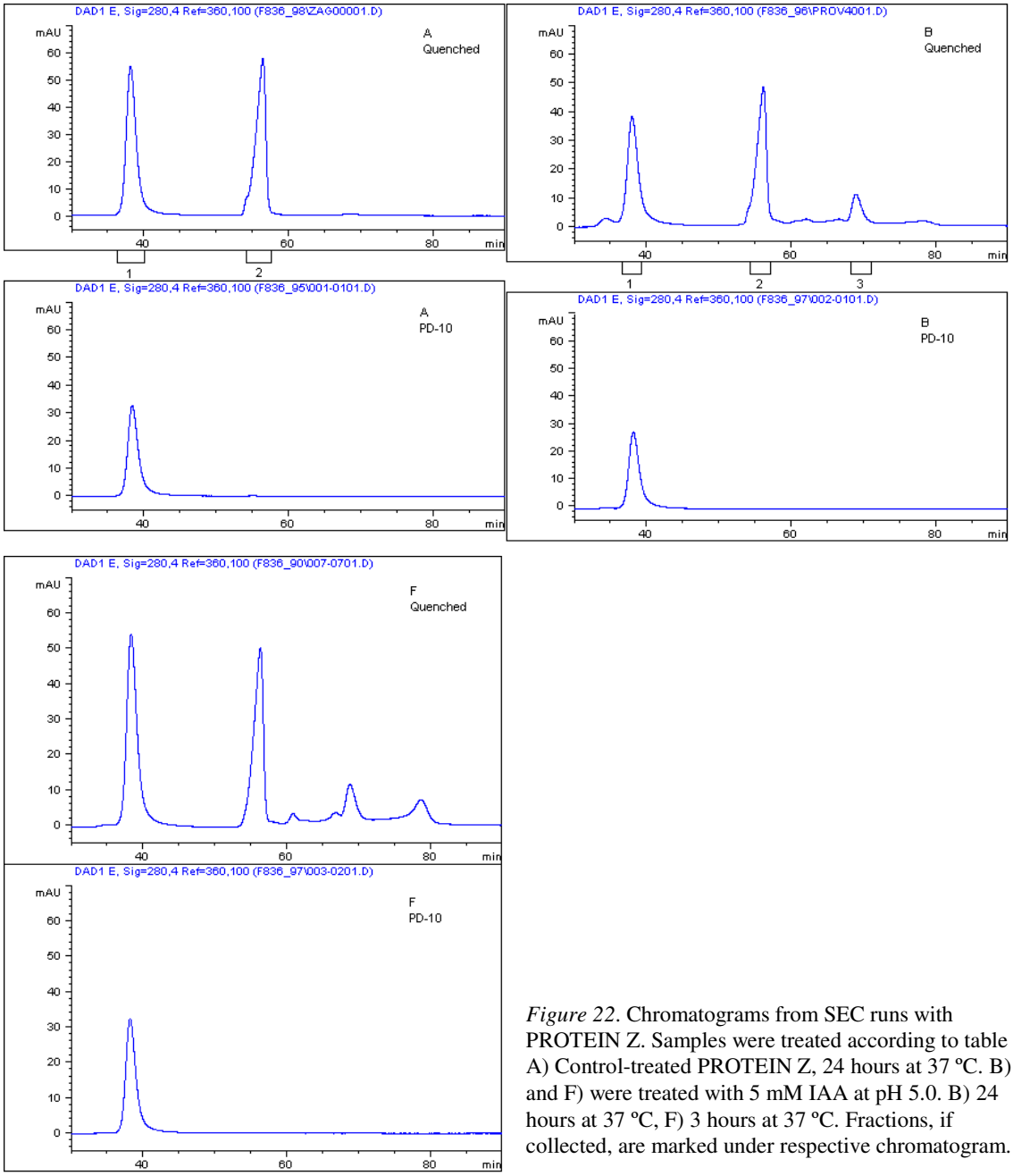

Figure 22. Chromatograms from SEC runs with PROTEIN Z. Samples were treated according to table 7. A) Control-treated PROTEIN Z, 24 hours at 37 ºC. B) and F) were treated with 5 mM IAA at pH 5.0. B) 24 hours at 37 ºC, F) 3 hours at 37 ºC. Fractions, if collected, are marked under respective chromatogram.

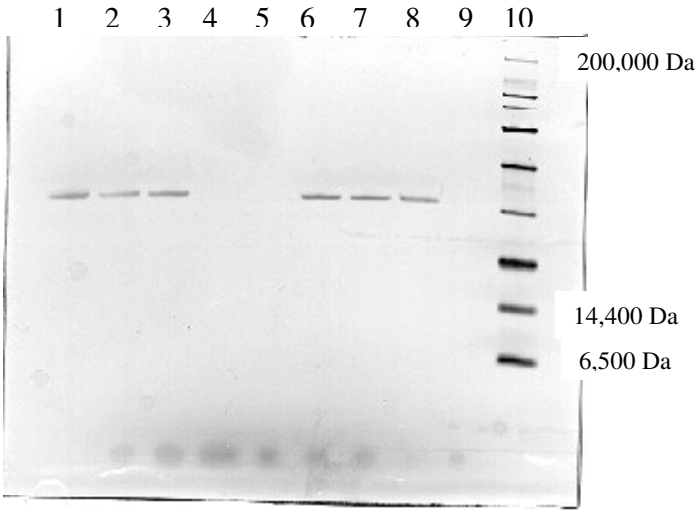

Figure 23. SDS-PAGE with NuPAGE® Novex Bis-Tris 10% pre-cast gels and silver staining. Positions 1-5. PROTEIN Z treated with 5 mM IAA for 24 hours at 37 ºC, pH 6.0. 1) Quenched sample, 2) PD-10 processed sample, 3)-5) Fractions 1, 2 and 3 collected during SEC run (figure 22). Positions 6)-9) Control-treated PROTEIN Z 24 hours at 37 ºC, pH 6.0. 6) Quenched sample, 7) PD-10 processed sample, 8) and 9) Fractions 1 and 2 collected during SEC run (figure 22), 10) Broad range marker.

6 Acknowledgements

First of all, I would like to thank my supervisor Christine Wesström for her great support when performing this project and writing the report. I would like to thank my supervisor Stig Johansson for his quick entrance at halftime, great support and good advice on how to write a report. I also want to thank everyone working in the process development group at Biovitrum AB, especially Sebastian Bauer, Sergei Kuprin and Maria Wrangel for their assistance. Thank you Peder Bergvall at Amersham Biosciences (Uppsala) for information and comments.

7 References

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Concentration determination using BIACORE

To determine the concentration of Anti-Apo AI and PROTEIN Z in different samples, standard curves were first obtained by running 10 samples of known concentration. The rate of formation of complex for a one to one reaction between analyte (Anti-Apo AI or PROTEIN Z) and ligand (Apo AI or Anti-PROTEIN Z) is given by:

$$\frac{dAB}{dt} = k_a \cdot [A] \cdot [B] - k_d \cdot [AB] \qquad (1)$$

, where [A] is the concentration of analyte, [B] is the concentration of free ligand, k_a is the association rate constant and k_d is the dissociation rate constant. In the BIACORE the response R is proportional to the concentration of complex [AB]. The maximum response R_{max} is the maximum analyte binding capacity. R_{max} is proportional to the concentration of ligand $[B_0] = [B] + [AB]$ and thus equation (1) can be written as

$$\frac{dR}{dt} = k_a \cdot C \cdot (R_{max} - R) - k_d R \qquad (2)$$

, where C is the concentration of analyte, which can be considered to be constant since analyte is constantly supplied in the flow channel. During the initial binding phase it can be assumed that the dissociation term can be neglected and thus the rate of formation of complex will be proportional to the concentration of ligand. However if the concentration of ligand is very high, the amount of ligand in complex will constitute such a small part of the total amount that the concentration of free ligand $[B] = R_{max} - R$ can be considered to be constant. The rate of formation of complex (initial binding rate) will then depend on the concentration of analyte. The initial binding rate is obtained from the sensorgram of a sample run (figure 1). Figure 2 shows an example of a standard curve for Anti-Apo AI. The concentration of unknown samples is calculated using the equation obtained from the standard curve (Method according to BIAapplications handbook, edition May 1994. Pharmacia Biosensor AB).

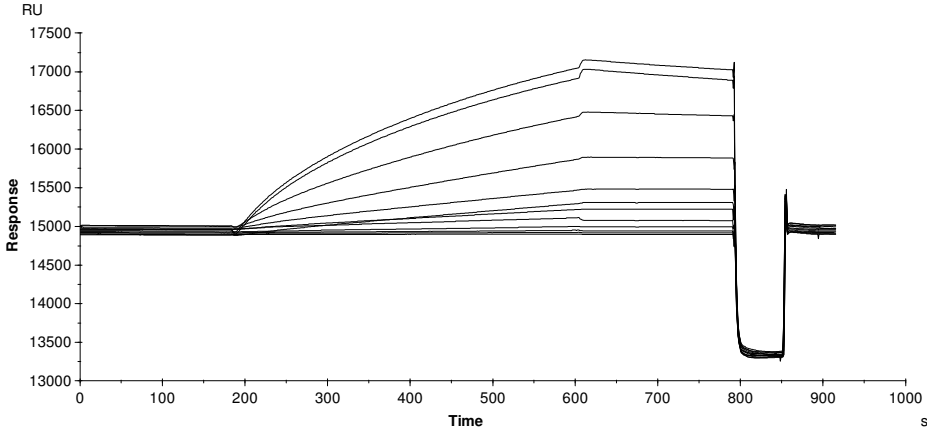

Figure 1. Example of a sensorgram obtained from a run with Anti-Apo AI standard.

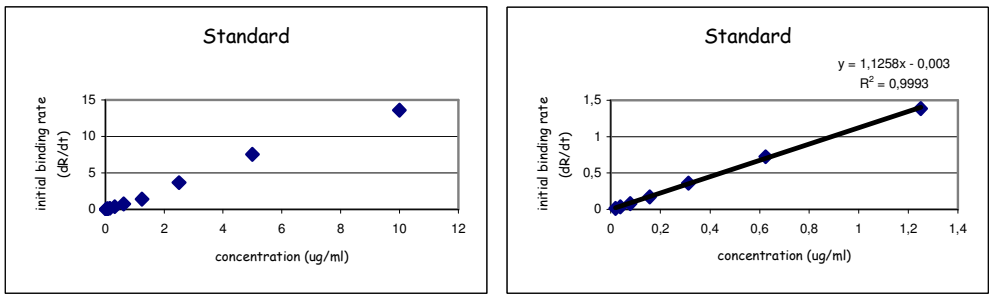

Figure 2. Example of a standard curve for Anti-Apo AI. The curve is only linear at low concentrations of analyte and the interval used for measurement is shown to the right.

Appendix 2

LRV of different viruses with INACTINE treatment

The conditions used in these inactivation studies were 0.1% INACTINE and pH 7.2. >= means below detection limit of the assay. N=no, Y=yes, RBCC=Red blood cell concentrate (reference 10).

Virus	Envelope	Sample	Start titer log	Time hours	T °C	LRV
Adeno	N	buffer	6.1	3	37	≥ 5.5
Adeno-2	N	RBCC			22	≥ 5.5
BPV	N	HSA, 59 mg/ml	5.4	6	22	1.4
BPV	N	HSA, 59 mg/ml	5.6	1	37	≥ 3.7
CPV	N	foetal bovine serum, 10%	?	6	22	3
CPV	N	plasma prot, 20 mg/ml + SD	5.8	6	25	3
CPV	N	HSA, 20 mg/ml	?	1	60	≥ 4.3
EMC	N	RBCC	6.2	6	22	2.5
EMC	N	plasma	6.3	6	30	2.0
EMC	N	buffer	6.2	6	37	≥ 5.7
Felini calici	N	plasma	8.1	1	30	6.1
FMDV	N	buffer	5.5	1	37	≥ 4.5
FMDV	N	RBCC			22	> 5.5
HAV	N	RBCC	5.5	6	22	1
HAV	N	plasma protein, 20 mg/ml	?	8	22	1
HAV	N	buffer	7.1	20	37	4.6
HAV	N	buffer	7.1	20	45	> 5.1
HAV	N	buffer	7.1	1	60	> 5.1
HAV	N	plasma	5.0	6	30, 37, 42	1
PCV	N	RBCC			22	< 2.5
Polio	N	buffer	6.0	1	37	1.0
Polio	N	buffer	7.3	6	37	2.7
Polio	N	buffer	7.3	12	37	5.0
Porcine entero	N	foetal bovine serum, 10%	?	4	22	≥ 4.4
Porcine entero	N	HSA, 20 mg/ml	?	1	60	≥ 4.8
PPV	N	buffer	6.5	3	22	≥ 6.0
PPV	N	IGIV, 50 mg/ml	6.2	6	22	3.6
PPV	N	IGIV, 50 mg/ml	6.2	6	22	2.7
PPV	N	IGIV, 50 mg/ml, dialysed	6.2	6	22	5.0
PPV	N	Plasma	6.2	5	22	4.0
PPV	N	PlasPlus	6.2	5	22	4.3
PPV	N	IVIG + SD	7.1	4	22	> 5.9
PPV	N	IGIV, 24 mg/ml	5.9	6	27	≥ 3.9
PPV	N	PlasPlus	8.4	6	30	≥ 6.5
PPV	N	plasma ± SD	7.7	3	30	≥ 6.5
PPV	N	plasma	7.8	3	30	≥ 4.7
PPV	N	fibrinogen, 10 mg/ml +SD	5.9	6	30	≥ 3.9

Virus	Envelope	Sample	Start titer log	Time hours	T °C	LRV
PPV	N	buffer	6.4	3	37	≥ 5.9
PPV	N	HSA, 59 mg/ml	9.5	2	37	≥ 5.9
Reo-3	N	IGIV, 47/mg/ml	6.6	1	22	≥ 4.2
Reo-3	N	RBCC			22	≥ 4.5
SV-40	N	RBCC	6.2	6	22	5.5
SV-40	N	buffer	5.6	6	37	4.4
VESV	N	RBCC			22	> 5.5
BVDV	Y	RBCC	5.7	6	22	≥ 5.2
BVDV	Y	plasma	7.7	3	30	≥ 6.5
BVDV	Y	buffer	5.3	6	30	≥ 4.8
BVDV	Y	plasma	4.2	1	30	≥ 3.7
BVDV	Y	plasma	7.7	3	30	≥ 6.5
CMV	Y	RBCC	5.2	6	22	≥ 3.7
HIV	Y	RBCC		6	22	1.2
HIV	Y	RBCC		8	37	≥ 5.0
HIV, lab adapted	Y	RBCC	5.8	6	22	1.2
HIV, lab adapted	Y	RBCC	5.8	24	22	4.9
HIV, lab adapted	Y	RBCC	5.8	48	22	≥ 4.8
HIV, lab adapted	Y	RBCC	5.7	24	30	≥ 4.8
HIV, lab adapted	Y	RBCC	5.2	8	37	≥ 4.8
HIV, lab adapted	Y	RBCC	6.1	6	37	5.5
HIV-clinical isolates	Y	RBCC	5.2	6	22	4.0
HIV-clinical isolates	Y	RBCC	5.2	18	22	≥ 4.8
HIV-clinical isolates	Y	RBCC	5.5	6	22	> 3.5
HIV-clinical isolates	Y	RBCC	4.8	6	22	> 2.8
HIV-clinical isolates	Y	RBCC	4.8	5	22	> 2.8
MULV	Y	RBCC	6.7	6	22	3.1
PRV	Y	RBCC	6.0	6	22	≥ 5.3
PRV	Y	culture media	?	6	22	3.7
PRV	Y	RBCC	8.0	6	22	6.0
PRV	Y	plasma	5.3	6	30	2.5
PRV	Y	buffer	8.1	3	37	≥ 7.5
PRV	Y	HSA, 20 mg/ml		1	60	≥ 4.8
Vaccinia	Y	buffer	9.1	6	37	≥ 6.9
VSV	Y	RBCC	7.9	6	22	≥ 7.3
VSV	Y	RBCC	8.1	3	22	≥ 6.9
VSV	Y	buffer	7.0	1	37	≥ 6.5

Appendix 3

LRV of different viruses with IAA treatment

Virus	Genome	Envelope	IAA mM	Time hours	Temp °C	Sample	Start log titre	Hold log red	LRV
BVDV	ss-RNA	+	5	4	23	50 mg IgG/ml, pH 5.0	8.1	0.4	4.4
BVDV	ss-RNA	+	5	8	23	50 mg IgG/ml, pH 5.0	8.1	0.4	> 5.8
BVDV	ss-RNA	+	5	2	23	50 NaAc, pH 5.0	5.5	-0.3	> 5.0
HIV-1	ss-RNA	+	5	2	23	50 mg IgG/ml, pH 5.0	7.3	-0.3	> 4.5
HIV-1	ss-RNA	+	5	8	23	50 mg IgG/ml, pH 5.0	7.3	-0.3	> 6.1
PRV	ds-DNA	+	5	1	23	50 mg IgG/ml, pH 5.0	4.5	1.4	> 3,9
PRV	ds-DNA	+	5	3	23	50 mM NaAc pH 5.0	5.6	0.5	3.9
PRV	ds-DNA	+	5	4	23	50 mg IgG/ml, pH 5.0	8.8	0.1	3.8
PRV	ds-DNA	+	5	8	23	50 mg IgG/ml, pH 5.0	8.8	0.1	5.4
PRV	ds-DNA	+	5	1	37	50 mg HSA/ml, pH 5.0	5.4	0.5	> 4,7
VSV	ss-RNA	+	5	< 0.5	23	50 mg IgG/ml, pH 5.0	6.5	1.0	> 5.8
VSV	ss-RNA	+	5	< 1	23	50 mM NaAc pH 5.0	5.9	2.8	> 6.1
BRV-1	ssRNA	-	5	2	23	50 mM NaAc, pH 5.0	6.3	0.7	> 5.8
CPV	ss-DNA	-	5	4	23	50 mg IgG/ml, pH 5.0	4.0	-0.1	> 3.3
EMCV	ss-RNA	-	5	2	23	50 mg IgG/ml, pH 5.0	7.0	0.6	> 6.3
HAV	ss-RNA	-	5	2	23	50 mg IgG/ml, pH 5.0	7.3	0.0	> 4.6
KRV	ss-DNA	-	5	2	23	50 mg IgG/ml, pH 5.0	5.0	0.1	> 4.3
MVM	ss-DNA	-	5	4	23	50 mg IgG/ml, pH 5.0	2,9	-0,3	> 3,2
PPV	ss-DNA	-	5	48	5	50 mg IgG/ml, pH 5.0	5.3	-0.2	> 4.6
PPV	ss-DNA	-	5	3	23	50 mg IgG/ml, pH 5.0	7.0	0.3	> 6.3
PPV	ss-DNA	-	5	4	23	50 mg IgG/ml, pH 5.0	7.0	0.3	< 4.6
PPV	ss-DNA	-	5	4	23	50 mM NaAc pH 5.0	6.5	0.4	> 5.8
PPV	ss-DNA	-	1	3	37	50 mg IgG/ml, pH 5.0	5.9	0.5	> 6.2
PPV	ss-DNA	-	5	0.05	60	50 mM NaAc, pH 5.0	5,9	0.3	> 5.4
Reo-3	ds-RNA	-	5	8	23	50 mg HSA/ml, pH 5.0	8.3	0.1	1.0
SVDV	ss-RNA	-	5	1	23	50 mg IgG/ml, pH 5.0	7.0	2.2	> 6.3
SVDV	ss-RNA	-	5	3	23	50 mM NaAc pH 5.0	5.8	-0.3	5.6

> = below detection limit of assay (reference 11).

Significance test, t-Test

The student's t-Test can be used to calculate if there is a significant difference between two experimental means. Two arrays of data, x_1 and x_2, have the means $\bar{x}_1$ and $\bar{x}_2$ with standard deviations S_1 and S_2. If the null hypothesis is assumed, the significance of $\bar{x}_1 - \bar{x}_2 \neq 0$ is tested. A statistic value *t* is calculated from the two means and compared with a critical value of *t* obtained from the *t* table. Depending on the degrees of freedom and the level of significance, there are different critical values of *t*. If the calculated value of $|t|$ is greater than the critical value, the difference between the two means is significant at that specific level.

The formula used to determine the statistic value *t* for two means with unequal variances was

$$t = \frac{\bar{x} - \bar{y}}{\sqrt{\frac{S_1^2}{n_1} + \frac{S_2^2}{n_2}}} \qquad (1)$$

where n_1 is the number of data points in array x_1 and n_2 is the number of data points in array x_2. The number of degrees of freedom (*df*) was calculated from the formula

$$df = \frac{\left(\frac{S_1^2}{n_1} + \frac{S_2^2}{n_2}\right)^2}{\frac{S_1^4}{n_1^2(n_1 - 1)} + \frac{S_2^4}{n_2^2(n_2 - 1)}} \qquad (2)$$

The statistic value t in the paired t-Test was calculated with the formula

$$t = \bar{d}\frac{\sqrt{n}}{S_d} \qquad (3)$$

where *n* is the number of pairs, $\bar{d}$ is the mean and S_d is the standard deviation of the difference *d* between the pairs. The number of degrees of freedom of *t* was *n* –1. (Miller, J.N. and Miller, J.C. Statistics and chemometrics for analytical chemistry Fourth edition, Pearson education Dorset 2000)