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Reducing sample complexity of polyclonal human autoantibodies by chromatofocusing

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ABSTRACT

Chromatofocusing was performed in order to separate a polyclonal antigen-specific mixture of human immunoglobulins (IgGs) that would then allow for further analyses of as few different IgGs as possible. Because polyclonal IgGs only differ by amino acid sequence and possible post-translational modifications but not by molecular weight, we chose chromatofocusing for protein separation by different isoelectric points. We isolated antigen-specific IgGs from commercially available intravenous immunoglobulins (IVIG) using a combination of affinity- and size exclusion-chromatography and in order to reduce the complexity of the starting material IVIG was then replaced by single-donor plasmapheresis material. Using two-dimensional gel electrophoresis (2-DE), we observed a clear decrease in the number of different light and heavy chains in the chromatofocusing peak as compared to the starting material. In parallel, we monitored slight problems with the selected peak in isoelectric focusing as the first dimension of 2-DE, displayed in by the less proper focusing of the spots. When we tested whether IgGs were binding to their specific antigen after chromatofocusing, we were able to show that they were still in native conformation. In conclusion, we showed that chromatofocusing can be used as a first step in the analysis of mixtures of very similar proteins, e.g. polyclonal IgG preparations, in order to minimize the amount of different proteins in separated fractions in a reproducible way.

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1. Introduction

Since intravenous administration of immunoglobulins (IVIG) is used as a therapy for patients with primary and secondary immune deficiencies, there is growing number of applications for these molecules ranging from inflammatory therapies to treatments for neurodegenerative diseases [1–4]. Plasma pools of 3000–10,000 donors are used to produce a batch of IVIG [5]. The mechanisms of action seem to influence several levels of the immune system but remain puzzling [6]. Human blood contains naturally occurring autoantibodies (nAbs) against several autologous proteins that can be isolated [7]. Therefore, passive immunization strategies as a therapeutic option in (auto-) antigen-linked diseases have been tested. Recently, nAbs directed against the β-amyloid peptide have been shown to be decreased in patients with Alzheimer's disease [8]. The therapeutic efficacy of IVIG in Alzheimer's disease is currently being tested in a phase III clinical trial [9]. Since the availability of IVIG is limited, the generation of monoclonal nAbs would be favorable, but would require isolation, separation and sequencing of antigen-specific nAbs as first steps.

Separation of proteins is a primary concern in proteomic research. Different proteome analyses of human body fluids (e.g. serum/plasma, cerebrospinal fluid etc.) have been performed in an attempt to discover biomarkers for diseases [10]. In order to reduce the complexity of serum/plasma samples, most of the highly abundant proteins (e.g. immunoglobulins, albumins etc.) are removed prior to proteome analysis by (immuno-) affinity chromatography using different resins and/or antibodies [11–16]. Afterwards, separation of the proteins is performed using several different analysis platforms – alone or in combination such as SDS-PAGE (one-dimensional and/or two-dimensional), P2DF and various kinds of ion exchange chromatography [17–21]. Although the separation of nAbs has a similar purpose as classical proteomic approaches, there are several different requirements: (i) one must perform highly reproducible separation of very similar proteins with the best feasible resolution (a condition that is quite contrary to classical proteomics), (ii) several milligrams of sample must be applied at a time, and (iii) proteins must be maintained in a native conformation – even after separation – for potential subsequent analyses. A good cost-benefit ratio would also be

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favourable. Two-dimensional gel electrophoresis (2-DE) is still the most important method for separating complex protein mixtures without losing information – e.g. post-translational modifications, which can affect the isoelectric point (p*I*) and/or the mass of a protein [22]. In the 2-DE separation of proteins according to their specific p*I* (which is, among other things, closely related to the amino acid composition) in the first dimension is coupled with SDS-PAGE as the second dimension. Because these specific classes of immunoglobulins share the same molecular weight (e.g. IgG ~150 kDa) differences in size cannot be used to distinguish various antibodies of the same class. Therefore, isoelectric focusing (IEF) seems to be a valuable tool for this purpose. In non-gel-based two-dimensional separation schemes, chromatofocusing (CF) separates proteins by their isoelectric points, as is done in the first dimension of 2-DE [23–26]. CF is a high-resolution chromatographic technique that forms an *in situ* descending linear pH-gradient [27]. The pH-gradient in separated fractions can reach <0.1 pH-units [28]. In contrast to 2-DE's gel-based IEF, CF has several advantages: namely, the proteins remain intact in solution and fractions can easily be assigned to distinct pH-values. The detection of eluting proteins by UV-spectrometry facilitates "fraction-matching" of several repeated runs even when high amounts of sample are applied, which increases reproducibility.

The aim of this work was to separate antigen-specific IgGs so as to allow further analyses (e.g. protein sequencing) of a mixture of as few different antibodies as possible. For this purpose, IgGs were isolated via affinity chromatography from commercially available IVIG preparations and later, as a first step toward reducing the sample complexity, from single-donor plasmapheresis material. We demonstrated that chromatofocusing – in combination with the use of single-donor material – significantly reduced the sample complexity of the fractions collected. Additionally, IgGs remained intact and were still able to bind to their specific antigen after separation.

Therefore, chromatofocusing is an appropriate tool for achieving high-resolution separation of proteins with the same molecular weight, prior to further analyses.

2. Materials and methods

2.1. Materials

Diethanolamine, glycine, hydrochloric acid, sodium dodecyl sulfate, trichloric acid, *N*,*N*,*N*′,*N*′-tetramethylethylenediamine (TEMED), Tris and urea were purchased from Carl Roth GmbH & Co. KG (Karlsruhe, Germany). AminoLink Plus resin, binding buffer, coupling buffer, elution buffer and Superblock were purchased from Pierce Biotechnology, Inc. (Rockford, IL, USA). All other chemicals were of analytical grade. Ultrapure water, obtained from the Milli-Q Aademic system (Millipore, Billerica, MA, USA), was used in the preparation of all solutions.

2.2. Purification of IgG from single-donor plasmapheresis material

Purification of IgG from single-donor plasmapheresis material was performed by protein G-affinity chromatography using a fast performance liquid chromatography (FPLC) system. After equilibration of the protein G-column with three column volumes of protein G binding buffer (Pierce, USA), 14 mg IgG (in a 50% protein G binding buffer-solution final concentration) per milliliter resin were applied. The column was washed with three column volumes of protein G binding buffer and antigen-specific IgG was subsequently eluted with seven column volumes of IgG elution buffer (Pierce, USA). The column effluent fractions were collected and pooled in 25% (v/v) 250 mM histidine buffer of pH 6.5. Absorbance was measured at 280 nm.

2.3. Affinity purification of antigen-specific IgG

In the affinity purification of antigen-specific IgG via a gravitation column, the antigen was coupled to an AminoLink Plus resin (Pierce, USA) at a concentration of 1 mg antigen/1 mL resin and washed thoroughly with 50 mL sterile PBS. 14 mg of Octagam (Octapharma, Germany) or purified single-donor IgG were allowed to bind to the coupled antigen. The column was washed extensively with 50 mL sterile PBS. Antigen-specific IgG was eluted through the addition of 8 mL 0.1 M glycine buffer at pH 2.8. The column effluent was collected in 500 μL fractions and subsequently neutralized by the addition of 25 μL 1 M Tris–HCl, pH 9. Fractions were dialyzed with PBS/0.05% NaN_3. All steps were performed at room temperature.

In addition, antigen columns were used as described above for the affinity purification of antigen-specific IgG via FPLC. After equilibration of the column with three column volumes of protein G binding buffer, purified single-donor IgG was applied at a concentration of 0.5 mg/mL resin. The column was washed with 20 column volumes of protein G binding buffer and antigen-specific IgG was subsequently eluted with 13 column volumes IgG elution buffer. The column effluent fractions were completely collected and pooled in 25% (v/v) 250 mM histidine buffer, pH 6.5. All steps were performed at a flow rate of 300 cm/h. Antigen-specific IgG pools were concentrated via VivaCell 100 (Sartorius, Germany), absorbance was measured at 280 nm and samples were filtered through a 0.22 μm filter (Millex GV 33 mm; Millipore, USA). Finally, samples were purified via size exclusion chromatography with PBS using a Superdex 200 prep grade column (GE Healthcare, UK) and stored in PBS/0.05% $NaNa_3$ at 4 °C until further analysis.

2.4. Chromatofocusing

Chromatofocusing resins and buffers were obtained from GE Healthcare (UK). 1.5 mg of affinity-purified IgG was equilibrated in 2 mL 25 mM diethanolamine–HCl (pH 9.5; starting buffer). The sample was applied to a 5 mm × 200 mm MonoP column previously equilibrated with 10 column volumes of starting buffer. The pH-gradient was developed with polybuffer 69, which was adjusted to a pH of 6 and a flow rate of 90 cm/h. 1 mL fractions of the column effluent were collected. The pH of each fraction was measured. The absorbance was measured at 280 nm. After the separation, bound protein was removed through a reverse wash of the column with 2 M NaCl. Collected fractions were precipitated with 10% trichloric acetic acid, washed three times with 90% ice-cold acetone to remove the ampholytes and stored at −20 °C until further analyses.

2.5. Analytical methods

2.5.1. One-dimensional SDS-PAGE analysis

The results of antibody purification were analyzed using 12% SDS-PAGE gels under non-reducing conditions (sample buffer without a reducing agent). Gels were stained with colloidal coomassie [29].

2.5.2. Two-dimensional gel electrophoresis (2-DE)

The 2-DE analysis was performed by Proteome Factory AG, Berlin, according to Klose and Kobalz [30]. Briefly, the antibody mixture was precipitated with 10% TCA and afterwards washed three times with 90% acetone. The air-dried pellet was resuspended in sample buffer (9 M urea, 2% ampholytes and 70 mM DTT). 20–70 μg of antibody solutions were loaded onto a 40 cm vertical rod gel (9 M urea, 4% acrylamide, 0.3% PDA, 5% glycerol, 0.06% TEMED and 2%

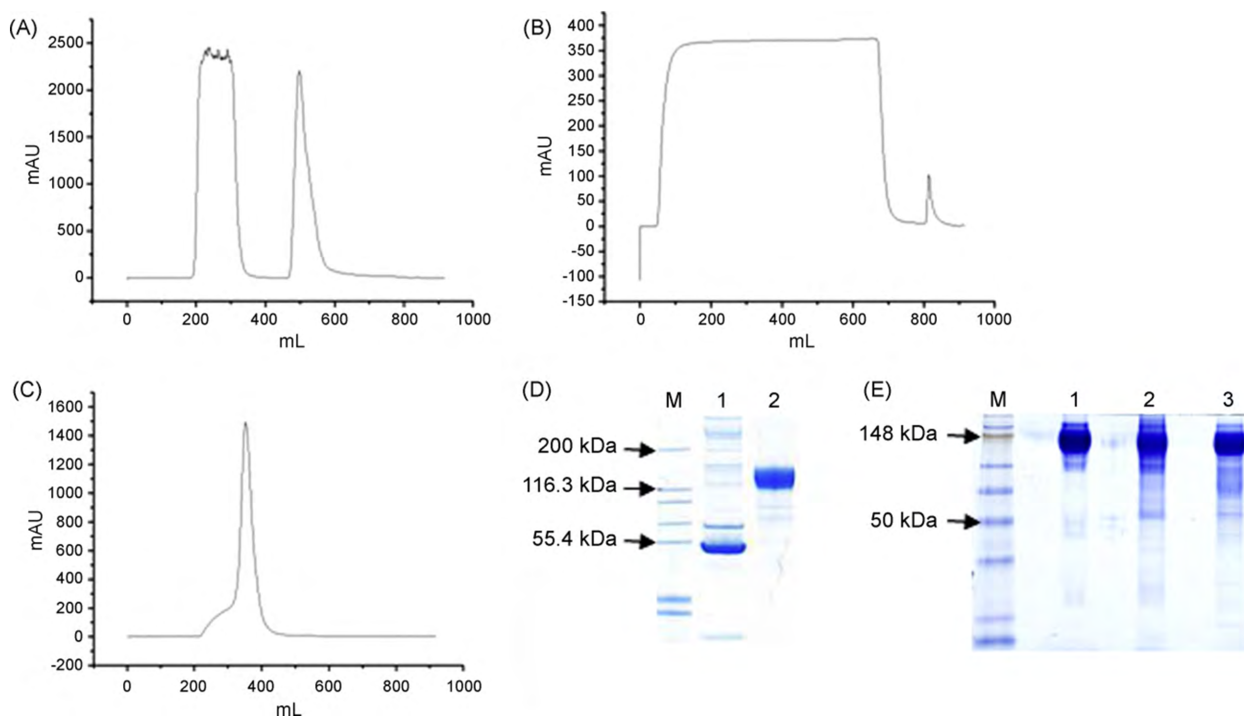

Fig. 1. Purification of antigen-specific IgGs. (A) Chromatogram of the IgG isolation from single-donor plasmapheresis material. (B) Chromatogram of the subsequent antigen-specific affinity purification. (C) Chromatogram of the following size exclusion chromatography. (D) SDS-PAGE analysis of the isolated material: 3 μg of total protein per sample were loaded on a 4–12% SDS-PAGE under non-reducing conditions; M: marker, lane 1: protein G flow through, lane 2: protein G eluate. (E) SDS-PAGE of IVIG (lane 1), antigen-specific IgGs from IVIG (lane 2) and antigen-specific IgGs out of single-donor material (lane 3); M: marker. 20 μg of total protein per sample were loaded on a 12% SDS-PAGE under non-reducing conditions.

carrier ampholytes (pH 2–11), 0.02% APS) for isoelectric focusing (IEF) at 12,820 V h in the first dimension. After focusing, the IEF gels were incubated in equilibration buffer containing 125 mM trisphosphate (pH 6.8), 40% glycerol, 65 mM DTT, and 3% SDS for 10 min and subsequently frozen at −80 °C. The second dimension SDS-PAGE gels (20 cm × 30 cm × 0.1 cm) were prepared with 375 mM Tris–HCl buffer (pH 8.8), 12% acrylamide, 0.2% bisacrylamide, 0.1% SDS and 0.03% TEMED. After thawing, the equilibrated IEF gels were immediately applied to two SDS-PAGE gels. Electrophoresis was performed at 140 V for 5 h. Afterwards, the gels were stained with MS compatible FireSilver (PS-2001; Proteome Factory, Germany).

2.5.3. Enzyme-linked immunosorbent assay (ELISA)

The enzyme-linked immunosorbent assay was performed as previously described [31]. Briefly, disposable round-bottom microtiterplates (IWAKI, Japan) were coated with 100 μL of the antigen solution (2.5 μg/mL in 0.9% saline) overnight at 4 °C. Non-specific binding was blocked with 300 μL of blocking buffer (Superblock; Pierce, USA) overnight at 4 °C. 100 μL of the first antibody in blocking buffer were added and incubated for 1 h at room temperature. Bound antibodies were detected with a goat anti-human IgG horseradish peroxidase (HRP) conjugate (0.3 μg/mL concentration; Calbiochem, Germany). A 100 μL aliquot of the HRP-substrate 3,3′,5,5′-tetramethylbenzidine (TMB; Calbiochem, Germany) was added and the reaction was stopped after 15 min using 2 M H_2SO_4. Absorbance was measured at 450 nm.

3. Results

3.1. Purification of IgG from single-donor plasmapheresis material as compared to commercially available IVIG preparation

Human IgG was isolated from single-donor plasmapheresis material by using self-packed protein G columns coupled to an FPLC system. After injection, the column was washed and the effluent was collected (Fig. 1A). Effluent fractions of repeated FPLC runs were pooled, filtered and concentrated. SDS-PAGE analysis of the pooled material primarily showed bands at a molecular weight of around 150 kDa (Fig. 1D), which is consistent with the molecular weight of human IgG. Bands of minor molecular weight were primarily observed in the flow through with only faint bands around 150 kDa (Fig. 1D), indicating good retention of IgG on the protein G-column during purification. Commercially available IVIG (99.6% human IgG; specification of the manufacturer), also showed a prominent band in the range of 150 kDa with faint bands in the lower molecular weight range (Fig. 1E).

3.2. Affinity purification of antigen-specific IgG from single-donor IgG and commercially available IVIG

In order to purify antigen-specific antibodies from the isolated IgG, affinity chromatography with an immobilized antigen was used. Purification was performed using a FPLC system for single-donor IgG and gravitation columns for IVIG, respectively. Fig. 1B shows a typical elution peak using the FPLC system (affinity purification via the gravity column also resulted in a typical elution curve not shown here). Peaks of repeated runs were collected, pooled, filtered and concentrated. In order to further remove smaller fragments, size exclusion chromatography of the concentrated samples was performed (Fig. 1C). SDS-PAGE analysis showed a prominent band at a molecular weight of around 150 kDa for antigen-specific purified samples from single-donor IgG and IVIG (Fig. 1E).

3.3. Two-dimensional gel electrophoresis of antigen-specific IgGs from single-donor IgG

In 2-DE, it is possible to discriminate between proteins according to two different properties. While the separation feature for

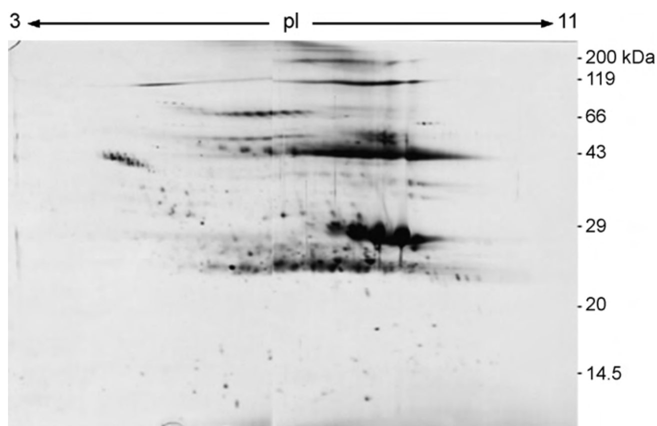

Fig. 2. Two-dimensional gel electrophoresis of antigen-specific IgGs. 70 μg of TCA–acetone-precipitated antigen-specific IgGs were separated by IEF in a 40 cm vertical rod gel with a linear pH-gradient from 2 to 11 – IEF gel was divided into two equally long parts after focusing – followed by SDS-PAGE in two vertical 12% gels. Protein detection was performed by silver staining.

IEF is the specific pI of each protein in the first dimension, a separation based on molecular weight follows as a second dimension. This combination is still the most feasible method for obtaining a high-resolution separation of complex protein mixtures. In order to demonstrate the complexity, 2-DE in antigen-specific IgGs was performed after affinity purification (Fig. 2). 70 μg of TCA–acetone-precipitated material were separated by IEF in a linear pH-gradient from 2 to 11. After reduction and alkylation, the second dimension was performed using a 12% SDS-PAGE. For protein detection, silver staining was used. The gel depicted a good pI-separation in the light chains of the antibodies (∼25 kDa) and – as expected – a "smear" in the molecular weight range of the heavy chains (∼60 kDa).

3.4. Chromatofocusing of antigen-specific IgGs results in defined fractions

Similar to IEF, the separation of proteins by chromatofocusing is based on different elution properties at different pH conditions due to differences in their amino acid sequences. Proteins remain in their native conformation and can thus be used for further functional analysis (e.g. ELISA, Western blot etc.). Antigen-specific IgG from IVIG and single-donor was separated on a linear pH-gradient from 9 to 6 and 1 mL fractions were collected (Fig. 3A and B). Fractions were tested for antigen binding by ELISA (Fig. 3C and D). The first peak is supposed to be due to the change from starting to elution buffer and showed no binding to the antigen (Fig. 3C and D).

3.5. Two-dimensional gel electrophoresis of defined chromatofocusing fractions show reduced complexity

Since 2-DE of antigen-specific IgG from IVIG showed a large number of different antibodies (Fig. 2), one chromatofocusing peak of the antigen-specific single-donor IgG was selected for further analysis of the sample complexity (Fig. 3B, marked peak). The respective fraction of the chromatofocusing run (∼20 μg total protein) was precipitated and further analyzed by 2-DE under the same

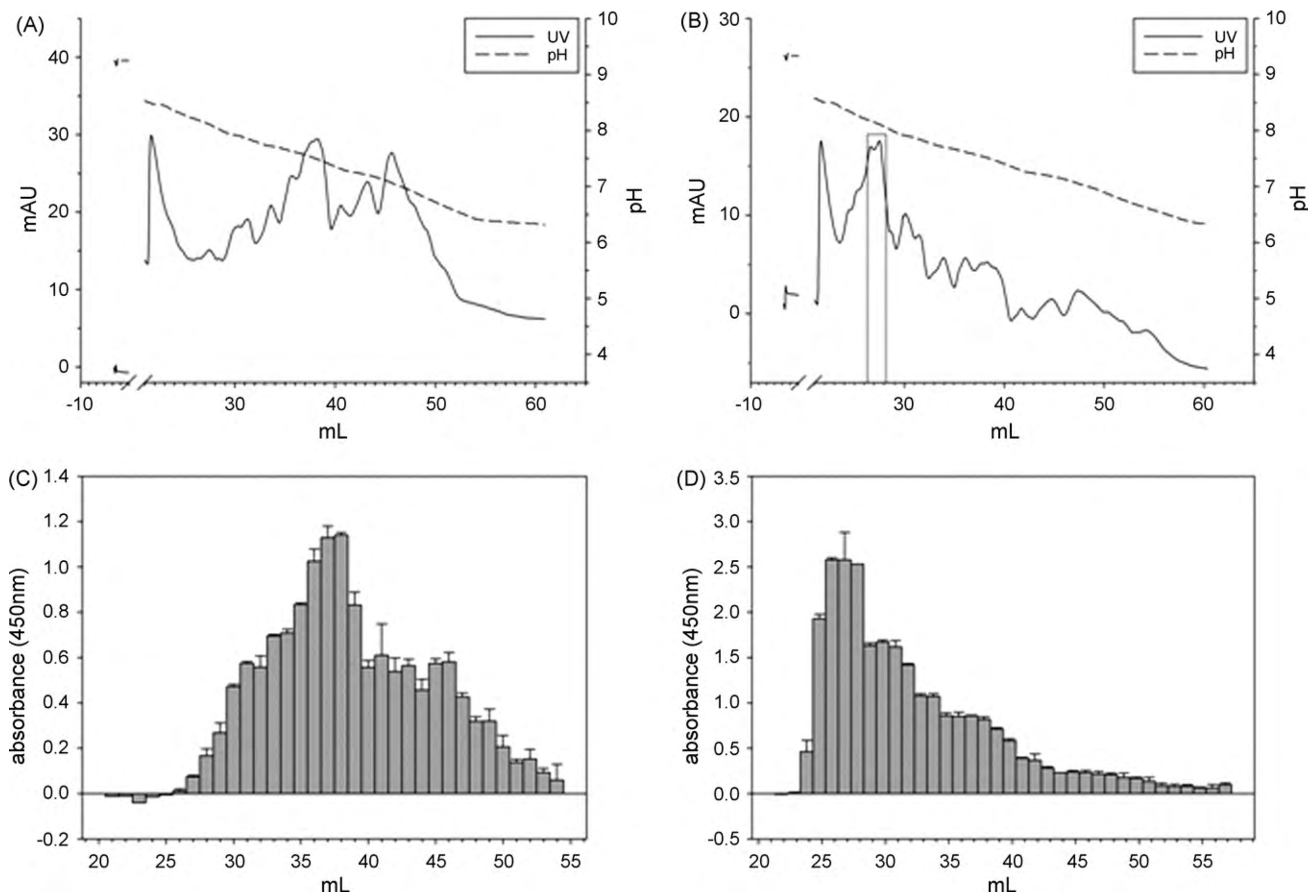

Fig. 3. Separation of antigen-specific IgGs by chromatofocusing. (A) Chromatofocusing chromatogram of antigen-specific IgGs from IVIG. (B) Chromatofocusing chromatogram of single-donor IgGs. 800 μg of IgGs were separated on a linear pH-gradient from 9 to 6 and collected in 1 mL fractions. (C and D) Antigen specificity of the collected fractions was determined by ELISA. For ELISA fractions of IgGs from IVIG (C) were diluted (1:5), whereas single-donor IgGs fractions (D) were used without dilution.

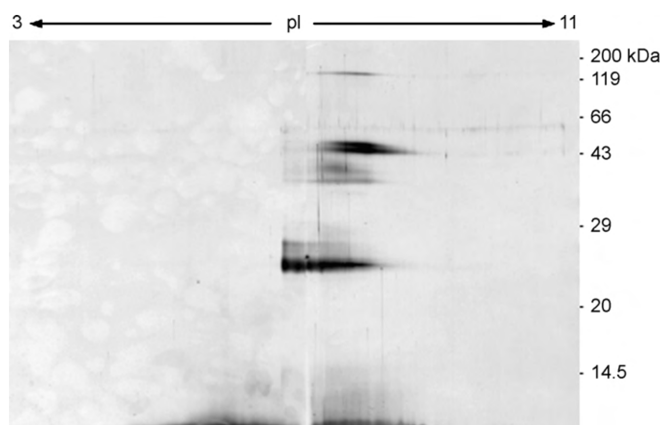

Fig. 4. Two-dimensional gel electrophoresis of a defined peak obtained by chromatofocusing of antigen-specific single-donor IgGs. 20 μg of TCA–acetone-precipitated antigen-specific single-donor IgGs of a defined chromatofocusing peak (marked fractions Fig. 3B) were separated by IEF in a 40 cm vertical rod gel with a linear pH-gradient from 2 to 11 – IEF gel was divided into two equally long parts after focusing – followed by SDS-PAGE in two vertical 12% gels. Protein detection was performed by silver staining.

conditions mentioned above (Fig. 4). For the light chains, a good separation in the neutral pH-range was observed, whereas there was a certain loss of IEF resolution in more basic pH-values. Separation of the heavy chains resulted in a protein-"smear" at a molecular weight of around 50 kDa.

4. Discussion

There are many different approaches to the separation of proteins in a complex mixture of biological fluids. Highly abundant protein species like albumins or immunoglobulins are usually depleted before analysis in order to reduce the sample complexity, to enrich low abundance proteins as well as to facilitate their separation [10]. But as immunoglobulins turned out to be potential diagnostic markers or therapeutics in human disease [7,8,32–34], they need to be isolated and separated for further characterization. In this study, we investigated the possibility of isolating antigen-specific IgGs from single-donor plasmapheresis material and reducing sample complexity through chromatofocusing, while maintaining IgGs in native conformation for further functional analyses.

Isolation and purification of antigen-specific IgGs from single-donor plasmapheresis material was performed using a combination of affinity chromatography via protein G (Fig. 1A), immobilized antigens (Fig. 1B) and size exclusion chromatography (Fig. 1C). Isolation of IgG via protein G-step was qualitatively comparable with commercial IVIG preparations, showing some faint bands in the range of 60–100 kDa in SDS-PAGE analysis (Fig. 1D). These bands may correspond to plasma proteins and/or antibody fragments due to the acidic elution. Todorova-Balvay et al. [35] demonstrated that immobilized metal–ion affinity chromatography (IMAC) is a suitable tool for the preparation of IgGs and their proteolytic fragments even under mild conditions (pH 7). Future research should test whether IMAC is a powerful alternative for the isolation of IgGs from human plasmapheresis material. SDS-PAGE of antigen-specific single-donor IgGs, IVIG and antigen-specific IgGs from IVIG revealed no major differences in sample purity (Fig. 1E). The main component was a band of around 150 kDa, consistent with the molecular weight of IgG. Both antigen-specific IgG preparations showed some faint bands between 50 and 100 kDa, most probably referring to antibody fragments resulting from the second acidic elution step.

We observed that antigen-specific affinity-purified IgGs from single-donor plasmapheresis material still show a high degree of complexity in 2-DE, as indicated by the separation of many different light chains at a molecular weight of ∼25 kDa (Fig. 2). This may be due to the fact that the purification steps via protein G and the immobilized antigen results in a polyclonal mixture of IgGs, since the starting material is a mixture of various proteins including antibodies against several antigens. Even 2-DE of monoclonal antibodies show a heterogeneous pattern [36] influenced by for example, different post-translational modifications and the oxidation state of amino acids. However, the separation of the heavy chains by 2-DE was not properly focused in IEF. Highly conserved domains are a major part of the heavy chain – especially in the Fc-part – and therefore fewer differences in the amino acid sequence and thus in the p*I* may be a possible reason for the problems observed in IEF. It is also known that IEF is becoming increasingly difficult at basic pH.

As a proof of concept, we first used antigen-specific IgGs from commercially available IVIG preparations for chromatofocusing because the availability of this starting material was less limited. Separation according to different p*I*s in a linear descending pH-gradient showed a characteristic chromatogram (Fig. 3A). By collecting the effluent in 1 mL fractions, we obtained a very good reproducibility in "peak-matching". Due to technically limited resolution of the pH-gradient [28], a wide peak base was observed. Kang and Frey [37] compared different column packing of strong and weak ion-exchange adsorbents for chromatofocusing with regard to the linearity of the pH-gradient and resolution. It was reported that strong ion-exchange adsorbents could achieve the same or even slightly better resolutions. MonoP, used in this study, is a weak ion-exchange adsorbent and therefore the resolution of the IgG separation probably may also be improved using strong ion-exchange adsorbents.

ELISA was used to estimate the ability to bind the specific antigen as this provides an indication of the native conformation and functionality of the specific IgGs (Fig. 3C and D). It is important to also mention that the ELISA is only a qualitative verification of functionality and does not reflect the quantity or the affinity of IgGs in the tested fractions.

The quality of the chromatograms obtained by chromatofocusing of antigen-specific single-donor IgGs was comparable to the chromatofocusing of antigen-specific IgGs from IVIG with respect to the linearity of the pH-gradient and resolution (Fig. 3B). Differences in peak distribution may be attributed to individual differences in the IgG pools of the starting material. A batch of IVIG consists of pools of 3000–10,000 donors [5] and consequently differences in the IgG pool of the donors are cleared. However, the fractions of antigen-specific single-donor IgGs that were collected were also able to bind to the specific antigen (Fig. 3D).

For further analysis of the complexity of the collected fractions we chose one peak that guaranteed the best and easiest reproducibility (Fig. 3B, marked peak). These fractions were pooled and precipitated to remove the polybuffer, which would have interfered with the IEF of the following analysis. 2-DE analysis of this the precipitated peak material showed a drastic reduction in sample complexity when compared to the complexity before chromatofocusing. A clear decrease in the number of light chains was observed, although the IEF in the first dimension was not as good as for the whole antigen-specific single-donor IgGs observable in spots that were not properly focused. With respect to the heavy chains, we also observed a reduction in the number, but because a weak IEF also occurred in the non-fractionated sample, it remains very difficult to discriminate between different heavy chains. Remaining traces of polybuffer after protein precipitation that can interfere with the IEF may offer one explanation for the worse resolution of the IEF after chromatofocusing. Kaplan et al. [38] recently demon-

strated that monoclonal light chains could differ in their isoelectric points in a range of up to 1.5 pH-units. In 2-DE, these differences would be detected as a smear. When we compared the isoelectric points of the selected peak (~8.2) with the IEF in 2-DE (Fig. 4), we observed a good correlation between IEF in 2-DE and chromatofocusing. It should also be noted that chromatofocusing was performed with the native proteins whereas in 2-DE the sample buffer contained DTT. Therefore, the light and heavy chains were not focused in a complex. This is shown in the different p*I*s of the light and heavy chains in the 2-D gel.

Chromatofocusing has numerous advantages as compared to 2-DE for separating proteins that are of the same molecular weight and have only slight differences in amino acid composition. It is a non-gel-based method, in which proteins remain in their native conformation and therefore are accessible for further analyses. Large amounts of protein can be applied without problems with resolution and detection of the separated proteins. Since proteins in chromatofocusing are detected by UV-spectrometry, no staining is necessary and the problem of signal saturation is eliminated. By varying the volume of the fractions collected, a high reproducibility in “peak-matching” can be obtained, ensuring a greater “purity” of the sample in case fraction pooling of serial chromatofocusing runs is necessary.

We were able to demonstrate through this study that chromatofocusing can be used to significantly reduce the sample complexity of single-donor IgGs. Therefore, chromatofocusing is a useful first step in the analysis of mixtures of very similar proteins, such as polyclonal antibody preparations, as a tool for minimizing the amount of various proteins in separated fractions in a reproducible way.

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