Journal of Chromatography B, 901 (2012) 34–40

Contents lists available at SciVerse ScienceDirect

Journal of Chromatography B

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

Comparison of displacement versus gradient mode for separation of a complex protein mixture by anion-exchange chromatography

Robert Ahrends[a,1], Björn Lichtner[a,1], Friedrich Buck[a], Diana Hildebrand[a], Marta Kotasinska[a], Oliver Kohlbacher[c], Marcel Kwiatkowski[a], Moritz Wagner[b], Maria Trusch[a], Hartmut Schlüter[a,*]

[a] University Medical Center Hamburg-Eppendorf, Department of Clinical Chemistry, Mass Spectrometric Proteomics, Campus Forschung, N27 Room 00.08, Martinistr. 52, 20246 Hamburg, Germany
[b] Agilent Technologies, Hewlett-Packard-Straße 8, 76337 Waldbronn, Germany
[c] University of Tübingen, Center for Bioinformatics, Quantitative Biology Center, and Department of Computer Science, Room C318, Sand 14, 72076 Tübingen, Germany

ARTICLE INFO

Article history:
Received 22 February 2012
Accepted 31 May 2012
Available online 6 June 2012

Keywords:
Displacement chromatography
Protein purification
Selected reaction monitoring
Mass spectrometry
Plasma proteins

ABSTRACT

Liquid chromatography is often the method of choice for the analysis of proteins in their native state. Nevertheless compared to two-dimensional electrophoresis, the resolution of common chromatographic techniques is low. Liquid chromatography in the displacement mode has previously been shown to offer higher resolution and to elute proteins in the high concentrations. In this study we compared to what extend displacement mode was a suitable alternative to gradient mode for the separation of a complex protein mixture using anion-exchange displacement chromatography and if it is therefore helpful for proteomic investigations. Hence we analyzed the qualitative protein composition of each fraction by tryptic digestion of the proteins, analysis of the tryptic peptides by liquid chromatography coupled to mass spectrometry followed by data base analysis and by measuring the elution profiles of 22 selected proteins with selected reaction monitoring mass spectrometry. In the fractions of displacement mode a significantly higher number of identified proteins (51 versus 16) was yielded in comparison to gradient mode. The resolution of displacement chromatography was slightly lower than of gradient chromatography for many but not for all proteins. The selectivities of displacement mode and gradient mode are very different. In conclusion displacement chromatography is a well suited alternative for top-down proteomic approaches which start with separating intact proteins first prior to mass spectrometric analysis of intact or digested proteins. The significant orthogonality of both modes may be used in the future for combining them in multidimensional fractionation procedures.

1. Introduction

In chromatography, four different modes of chromatography frontal, isocratic, gradient, and displacement are known. The displacement mode was already introduced in 1943 by Tiselius and was almost from the beginning on used for the separation of proteins from complex mixtures [1,2]. Until today, protein displacement chromatography has not yet been applied to proteomics for the separation of highly complex protein mixtures but it was previously shown to work very efficiently in proteomics as first dimension for peptide separation [2,3]. Recently Cramer and colleagues published a work that underscores the capability of displacement chromatography to enrich low abundant proteins out of multi component test mixtures [4]. Displacement chromatography is based on competitive binding of the sample components themselves as well as an additional molecule, which is added to the eluent, the displacer. The column is first equilibrated with a sample application buffer, the "carrier". This carrier has to support high affinities of the sample components for the stationary phase. Usually the composition of the carrier is identical with the sample application buffer in gradient elution chromatography. During sample loading, the sample components compete among themselves for the binding sites of the stationary phase. The component with the highest affinity to the stationary phase binds to the chromatographic material at the top of the column, displacing components with lower affinities from their binding sites. This process can be described as sample displacement [5]. Directly after sample loading, the displacer-containing eluent is pumped onto the column. Usually the displacer is dissolved in the carrier. It is mandatory that the displacer has a very high affinity for the stationary phase, ideally a higher affinity than any of the sample components. As soon as the displacer molecules adsorb at the stationary phase, they displace all sample components on top of the column. These

* Corresponding author. Tel.: +49 40 7410 58795; fax: +49 40 7410 40097.
E-mail address: hschluet@uke.de (H. Schlüter).
[1] Equally contributed.

1570-0232/$ – see front matter © 2012 Elsevier B.V. All rights reserved.
http://dx.doi.org/10.1016/j.jchromb.2012.05.037

sample molecules, having the highest affinity towards the stationary phase, displace their neighboring sample components having a lower affinity. Each displaced component acts as a displacing agent for neighboring components of lower affinity for the stationary phase. Thus the displacer enforces a competition reaction which spreads down the column resulting in isotachic movement of all bands with the speed of the displacer front. By loading continuously displacer molecules a system of contiguous zones, termed "displacement train," will move down the column [6]. Within the displacement train the sample components are arranged in the order of their affinity to the stationary phase, with the component having the lowest affinity to the stationary phase at the head of the displacement train and the most strongly retained component directly in front of the displacer. Provided that the column is sufficiently long and the components are present in abundant amounts, each zone contains ideally only one component in high purity [7]. The displacement chromatography is finished if the stationary phase is saturated by the displacer resulting in the elution of significant increased amounts of displacer.

Displacement chromatography differs from elution chromatography in several points: by utilizing non-linear isotherms versus linear isotherms, by yielding slightly overlapping bands versus base line separation peaks; however, the most notable difference is that in displacement chromatography much higher sample feeds are possible. Furthermore the displacement elution mechanism can avoid concentration dependent protein precipitation since protein concentration reaches a plateau of adjustable concentration, while gradient elution protein chromatography is often plagued by concentration maxima. Different examples have already demonstrated that displacement chromatography is well suited for protein purification. Cramer and colleagues were able to show that the displacement elution mode can be used in combination with hydrophobic interaction, reversed phase and ion-exchange chromatography [8–10].

The main goal of our study was to investigate if ion-exchange displacement chromatography is providing advantages for separation of complex protein mixtures in comparison to gradient chromatography. In this study blood plasma protein fraction Cohn IV-4 was chosen as a sample with a complex protein composition. Cohn fractions are yielded from frozen blood plasma by ethanol precipitation at a specific pH, ionic strength, temperature and protein concentration. The Cohn method [11] has developed into a well-established industrial process over the decades, capable of isolating a wide variety of clinically helpful products [12,13]. The Cohn fraction IV-4, which is yielded from precipitation of plasma proteins with 40% ethanol at a pH between 4.48 and 5.42, contains over 80 proteins whereas the main abundant proteins are transferrin alpha- and beta-globulins, apolipoprotein A-I and ceruloplasmin [3].

Furthermore the aim of this study was to answer the question if DM is more beneficial in comparison to GM for the analysis of proteomes with respect to protein species following the top down strategy approaches which implies the separation on the level of proteins prior to analysis of intact protein species by high resolution mass spectrometry [14] or to analysis of their tryptic digests with liquid chromatography coupled to tandem mass spectrometry (LC–MS/MS). Protein species are formed by post-translational modification of the side chains of proteins or by their truncation [15–18].

2. Experimental

2.1. Method

A plasma protein fraction (Cohn fraction) was chromatographed with an AEX chromatography in the gradient mode and in the displacement mode. The proteins in the resulting fractions (2×30 fractions) were digested with trypsin. Selected tryptic peptides from each of the fractions were analyzed with a Q-TOF Premier Waters (Manchester, UK) mass spectrometer and Agilent HPLC-Chip coupled to a 6410 triple quadrupole mass spectrometer (Santa Clara, USA) in the SRM mode. The mass spectrometric data from the Q-TOF instrument were processed by a set of bioinformatic tools applying stringent criteria for guaranteeing high confidence concerning the identity of the proteins.

2.2. Chemicals

Plasma protein Cohn fraction IV-4 and phosphate buffer was purchased from Sigma–Aldrich (St. Louis, USA). Trypsin was obtained from Promega (Madison, USA). HPLC-grade water and HPLC-grade acetonitrile were purchased from Baker (Deventer, Netherlands). Sodium hydrogen carbonate was purchased from Merck (Darmstadt, Germany). Formic acid was purchased from Fluka (St. Louis, USA). Chondroitin-4-sulfate was obtained from Calbiochem (San Diego, CA, USA).

2.3. Protein separation

2.3.1. AEX chromatography

Both types of separation were carried out on an Mini Q 3.2/3 (strong AEX column, GE Healthcare, Little Chalfont, UK, 3.2 mm × 30 mm, 3 μm) using a Smart system (Pharmacia Biotech, Uppsala, Sweden) for chromatography. The protein elution profile was monitored online by UV and by conductivity.

2.3.2. Gradient elution mode (GM)

For the gradient elution mode the following conditions were used: Injected sample amount: 100 μg dissolved in 50 μl buffer A (20 mM phosphate buffer, pH 7), equilibration, loading and separation flow rate 100 μl/min. After the column was equilibrated for 10 min the sample was loaded and eluted using eluent B (1 M NaCl in 20 mM phosphate buffer, pH 7) by the following gradient: 0–40% B in 34 min, 40–100% B in 3.4 min. Using UV detection at 220 and 280 nm respectively the eluting proteins were monitored and collected with a fraction size of 150 μl, subsequently desalted, reduced, alkylated and trypsinated for further investigation.

2.3.3. Displacement mode (DM)

For DM 800 μg of sample was dissolved in 200 μl buffer A (20 mM phosphate buffer, pH 7) which was also applied for equilibration and loading. Separations were carried out with a flow rate of 10 μl/min. After the column was equilibrated for 10 min, the sample was loaded and the mobile phase shifted to the displacer containing solution B (20 mM phosphate buffer, pH 7, 10 mg/ml chondroitin-4-sulfate) to elute the proteins. The eluting proteins were monitored using UV detection at λ 280 nm and conductivity. The sample were collected with a fraction size of 150 μl, subsequently reduced, alkylated, trypsinated and desalted for further investigation.

2.3.4. Proteolytic digestion

For tryptic digestion 25 mg of Cohn IV-4 was reduced using 200 μl 6 M urea, 20 μl of a 200 mM dithiothreitol and 100 mM $NaHCO_3$ buffer (pH 8.3) for 1 h at 25 °C. Alkylation was carried out at 25 °C for 1 h by adding additional 140 μl of 100 mM iodacetamide, dissolved in an aqueous 100 mM $NaHCO_3$ (pH 8.3) buffer. Then, to stop the alkylation reaction 40 μl of the 200 mM dithiothreitol and 100 mM $NaHCO_3$ buffer (pH 8.3) were added and again incubated at room temperature for 1 h. Thereafter 1700 μl of a 100 mM $NaHCO_3$ buffer (pH 8.3) were added and the mixture was transferred into

a glass vial. 50 μl of trypsin (0.25 μg/μl dissolved in trypsin resuspension buffer, Promega) were added (enzyme/protein, 1:100). The reaction mixture was incubated for 12 h at 37 °C. The reaction was quenched by adding formic acid to a final concentration of 0.1%.

2.3.5. Desalting

For desalting, 2 ml of the tryptic digested sample solution was injected onto a HPLC system (Äkta Explorer 100, GE-Healthcare). The peptides were desalted via a reversed phase column (Lobar® 240-10 LiChroprep® 10 mm × 240 mm; Merck, Darmstadt), equilibrated with 0.2% formic acid in HPLC-grade water. After binding the tryptic peptides on the stationary-phase and washing them with 28 ml water 0.2% formic acid, the peptides were desorbed from the stationary-phase with an acetonitrile gradient (0–100% B in 10 min; eluent B: 60% acetonitrile in water). A flow rate of 1.5 ml/min was used. The fraction within the acetonitrile gradient showing a significant UV-absorption at 220 nm and eluting within the gradient was collected.

2.3.6. Protein identification

For the discovery approach a nano-Acquity UHPLC (Waters, Manchester, UK) hyphenated to a Q-TOF Premier™ (Waters, Manchester, UK) was used. The sample trapping was carried out on a Symmetry® C18 column (20 mm, 180 μm, 5 μm) and the peptide separation on a BEH130 C18 column (100 mm, 100 μm, 1.7 μm). The samples were loaded with a flow rate of 5 μl/min buffer A (0.1% formic acid, 99.9% H_2O) to the precolumn. Separation was carried out at 0.4 μl/min flow and a linear gradient ranging from 3 to 50% buffer B (99.9% ACN 0.1% FA) in 90 min. For nano-spray the capillary voltage was set to 1800 V, a source temperature of 80 °C, a curtain gas of 31 l/h and nanoflow gas pressure of 0.34 bar was applied. The Q-TOF was controlled by MassLynx™ 4.1 software, for CID MSE fragmentation a collision energy ramp ranging from 20 to 42 V was used and MS/MS spectra were obtained in the mass range from 100 to 1600 *m*/*z*.

The protein identification was carried out on ProteinLynx™ by an adjustment of MS and MS/MS (MSE) spectra and a final search of the precursor and fragment ions against the SwissProt database (version 56.8) for human proteins. Only proteins with a reported probability score of at least of 100 (ProteinLynx™), which were covered by at least two distinct peptides were used to confirm the protein identity and kept for further analysis.

2.3.7. Protein quantification

The protein quantification was carried out on an Agilent 6410 triple quadrupole mass spectrometer (Agilent Technologies, Santa Clara, CA, USA) coupled to a HPLC-Chip system (Agilent Technologies, Waldbronn, Germany) which was connected to a HPLC system (nanoHPLC 1200 system, Agilent Technologies, Waldbronn, Germany) for separation. The HPLC chip contained two channels both filled with a reversed phase material (Zorbax 5 μm 300SB-C18; 40 nl volume filled with chromatographic material for trapping; a channel with 150 mm × 75 μm filled with chromatographic material for separation). Sample loading (8 μl/sample) from the sample vial plate into the enrichment column was performed at a flow rate set to 4 μl/min with the mix of the two following mobile phases at a ratio 97:3 (mobile phase A: 0.2% formic acid in H_2O; mobile phase B: 100% ACN). LC gradient was delivered with a flow rate of 400 nl/min. Tryptic peptides were eluted from the reversed phase column into the mass spectrometer using a linear gradient elution of 3–15% B in 8 min, 15–45% in 30 min and 45–60% in 3 min, the post time was 4 min.

For the SRM analysis a triple quadruple MS with the following condition was used: Drying gas 5 l/min, 325 °C, spray voltage of 1850 V and unit resolution (±0.7 Da) for Q1 and Q3, to calculate the initial collision energy for each peptide (*m*/*z* signal) and to optimize each transition peptide optimizer (Agilent, Santa Clara, CA, USA) was used. In this study protein derived peptides serve as probes to monitor the elution of targeted proteins during AEX separation. To optimize peptide transition parameters (fragment ion transition derived from one precursor ion) a digested, un-separated Cohn 4-IV fraction was used in LC/ESI SRM experiments. After setting up an initial protein target list, each transition was optimized online by varying the collision energy from 16 to 36 V in one-V steps (Suppl. Table 1). The optimization procedure generates the parameters set which is needed to detect the protein derived peptides with the best possible sensitivity during the subsequent following quantification steps. To quantify the proteins by their derived peptides in DM or GM each fraction was trypsinated and the protein derived peptides of each fraction quantified by SRM over the time course of elution (all fractions, one by one). To test the reproducibility (Suppl. Fig. 3) of our analysis one fraction was analyzed in quadruplicate over the duration of 30 h were our AEX fractions were analyzed.

3. Results and discussion

To investigate if the displacement chromatography elution mode (DM) is an alternative for protein separation in comparison to the gradient elution mode (GM), the proteins of the Cohn IV-4 fraction were separated on an AEX column using DM and GM. Fig. 1 represents a scheme of the workflow. Each of the resulting fractions was subjected to tryptic digestion and desalting. The tryptic peptides of each fraction were analyzed either for protein identification or for quantification of 22 selected proteins. For identification of the proteins in the individual fractions a proteomics workflow was applied, briefly the peptides eluting into the mass spectrometer were analyzed with a tandem mass spectrometer and the resulting mass spectrometric data were compared with a protein data base by a search engine. From the list of identified proteins 22 proteins were selected representing high, middle and low abundant proteins for studying their elution behavior by quantifying their relative amounts in each of the fractions. Relative quantification of the 22 target proteins was performed by analyzing the tryptic peptides by LC–MS selected reaction monitoring (SRM) approach. SRM experiments were introduced in the late seventies [19], using triple quadrupole mass spectrometers and are commonly used to quantify small molecules and was recently introduced for protein quantification [20]. Briefly, for quantification of a target protein it must be digested by trypsin first. As representative of the target protein two or more tryptic peptides are selected. For these peptides SRM parameters are determined, the transitions. The precursor mass is used for selecting the peptide ion of interest by the first quadrupole of the mass spectrometer. The isolated peptide ion is then fragmented in the second quadrupole. A defined fragment ion is isolated by the quadrupole and thereafter hits the detector. Since the triple quadrupole mass spectrometer is coupled to a HPLC column a chromatogram is obtained for the selected peptide. A triple quadrupole is a fast scanning instrument, therefore many peptides can be detected in parallel within one HPLC run.

3.1. Investigation of elution modes by monitoring UV and conductivity

AEX chromatography in GM and DM were monitored online by UV and conductivity detection. As shown in Fig. 2, both types of elution modes display different elution profiles (Fig. 2A and B). In GM, typically Gaussian peak shapes are present in the chromatogram, with a group of peaks nearly base-line separated (Fig. 2B), whereas in DM the UV profile was characterized by an irregular shape, which did not necessarily imply an effective separation, so long as the profile was interpreted with normal criteria for gradient

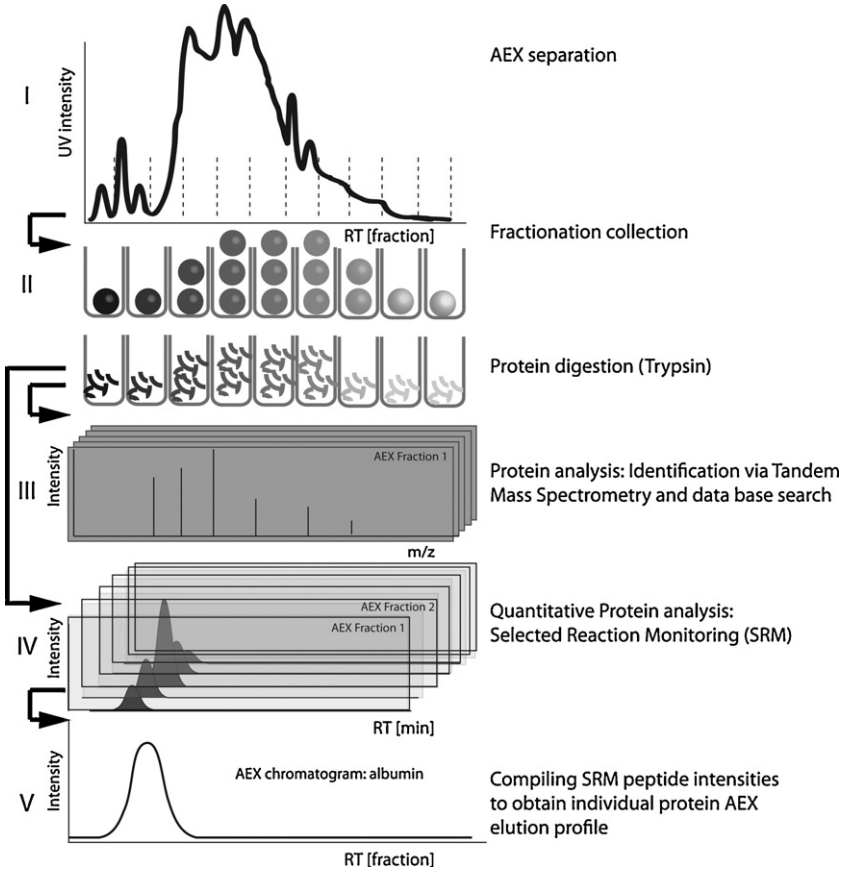

Fig. 1. Workflow of the analysis of gradient mode and displacement mode AEX chromatography fractions to identify proteins and to construct AEX chromatography elution profiles of individual proteins. The plasma protein fraction Cohn IV-4 is separated by DM or GM (I). Individual fractions are collected, reduced, alkylated and digested (II). The trypsin hydrolyzed proteins are then identified based on their derived peptides by LC–MS/MS experiments and subsequent data base search, which deliver the qualitative information how many proteins are in each fraction (III). The mass spectrometric data of the tryptic peptides of 22 selected proteins were used to develop SRM experiments for the quantitation of the selected proteins. Each AEX fraction was analyzed with SRM (IV). The SRM data were used for reassemble the elution profiles for 22 individual proteins (Fig. 4, Suppl. Figs. 1 and 2).

chromatography (Fig. 2A). The UV and conductivity profile reached a plateau at 70 min, indicating the elution of the displacer.

3.2. Qualitative analysis of the protein compositions of the gradient and displacement AEX chromatography fractions by LC–MS

Thirty fractions per chromatographic mode were collected. Proteins in each fraction were digested with trypsin and tryptic peptides analyzed by LC–MS/MS followed by data base search. In total, 16 proteins in the fractions of the GM chromatography and 51 proteins in the fractions of the DM chromatography were identified (Suppl. Table 2). The significant difference between GM and DM can be explained by significantly larger protein concentrations in DM fractions: Generally, in protein identification via the LC–MS/MS approach the number of identifiable proteins increase with increasing protein concentrations. The bar graph chromatograms of Fig. 3 display the number of identified proteins in each fraction for these two modes. Here, GM (Fig. 3B) demonstrated a more even distribution whereas the DM displayed an increasing number of identified

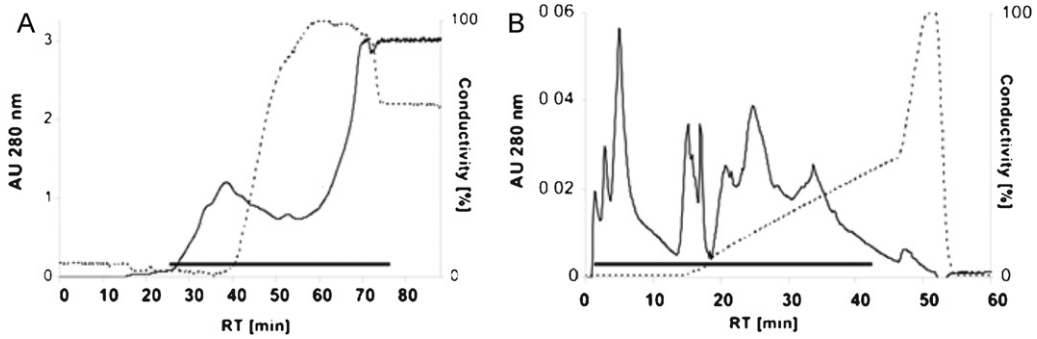

Fig. 2. AEX chromatograms of protein separation applying displacement or gradient modus. Continuous line: UV absorption at λ = 280 nm, dashed line: Conductivity. Bold black line: time of fractionation (A) AEX applying displacement modus. The separation was carried out using 800 μg of protein dissolved in 200 μl 20 mM phosphate buffer pH 7.0, flow rate 10 μl, 25 °C and the displacement solution (10 mg/ml chondroitin-4-sulfate) which was pumped continuously to the column. (B) AEX separation in GM was carried out using 100 μg protein of Cohn fraction IV-4 dissolved in 50 μl 20 mM phosphate buffer pH 7.0. The proteins were separated by applying a flow rate of 100 μl/min at 25 °C and a gradient up to 1 M NaCl in 20 mM phosphate buffer pH 7.0 (gradient: 0–50% in 24 min, 50–100% eluent in 3.4 min).

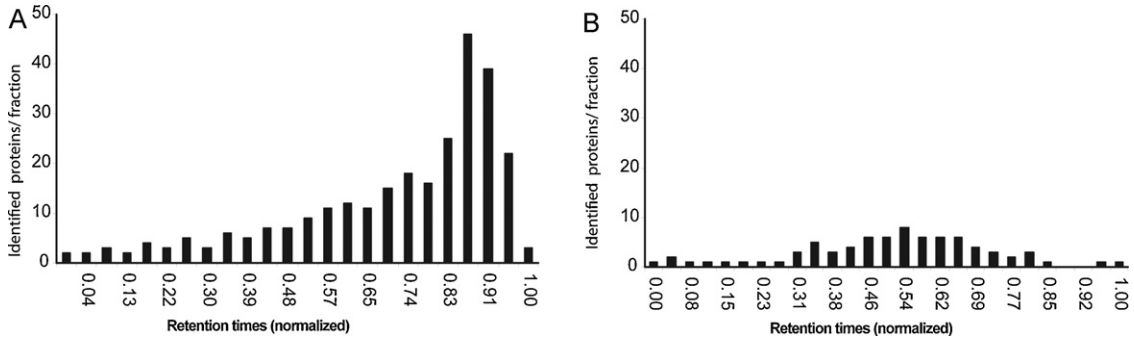

Fig. 3. Number of identified proteins applying gradient or displacement modus. The proteins of every fraction were digested by trypsin. The tryptic peptides were analyzed by LC–MS using a Q-TOF mass spectrometer. For MS analysis using a Q-TOF instrument the amount of protein derived out the two separations was adjusted to guarantee that an equal amount of peptides is analyzed by LC/ESI MS. (A) Number of identified proteins per fraction applying displacement mode. (B) number of identified proteins per fraction applying GM, since different times were used here for the sample collection the retention time was normalized.

proteins at the end of the displacement run. This observation can be explained by the large number of plasma proteins, which are negatively charged at pH 7 and therefore bind to the AEX. The majority of plasma proteins in the Cohn IV-4 fraction has p*I* values significantly below 7 [3,21]. In Fig. 3A the elution behavior typical for displacement chromatography became obvious. The increasing number of identified proteins correlates with increased concentration of the proteins and increased elution time. The final decrease of the number of identified proteins at DM was caused by the increasing concentration of the displacer, which co-eluted in the last three fractions (0.9–1 normalized RT in Fig. 3A; 67–75 min in Fig. 2A) with the proteins. The diagrams clearly signified a major advantage of the DM, which yielded much higher protein concentrations in the eluting fractions of DM than of GM.

This can be a possible explanation why out of the DM 3 times more proteins are identified by qualitative MS then out of the GM. The concentration enhancement effect at the DM and possible ion suppression effect at GM resulted finally that more proteins being identified out of the fraction of the DM AEX separation.

3.3. Quantitative analysis of selected proteins of the gradient and displacement chromatography fractions by LC–MS

The qualitative analysis by the LC–MS approach described above allows a rough estimate of abundance of each identified protein, however a profile of individual proteins cannot be deduced from these data (Fig. 3, Suppl. Table 2). For studying the elution profiles of individual proteins 22 proteins were chosen covering different levels of abundance and for each protein selected reaction monitoring (SRM) mass spectrometry based relative quantification method was developed. SRM performed on modern triple-quadrupole instruments provides the opportunity to relatively quantify a larger set of proteins within one single LC–MS run. SRM methods are very selective, sensitive and reproducible (Suppl. Fig. 3). Furthermore these instruments offer a broad dynamic range for most of the analytes [20]. For analyzing the success of chromatographic separations analysis of the fractions with SDS-PAGE is common [22]. However, qualitative and semi-quantitative analysis of target proteins by SDS-PAGE is possible only by western blots. By Coomassie-stained SDS-PAGE analysis of chromatographic fractions only very rough estimations are possible concerning qualitative and quantitative aspects since the bands usually represent mixtures of proteins. In addition from many single genes several protein products (protein species) are present in biological systems [16,23]. The advantage of SRM in comparison to SDS-PAGE is that SRM provides a high confidence regarding the identity of the targeted proteins. Even different protein species originating from one single gene are detectable with SRM. For relatively quantification of the chosen proteins by SRM 2 or 3 of their tryptic peptides per protein were selected (Suppl. Table 1) from the LC–MS/MS data of the analysis of the qualitative composition of proteins and from previous LC–MS/MS data [3], resulting in an transition list (Suppl. Table 1). In total, 166 transitions from 58 peptides were used to quantify 22 proteins. The elution profiles of individual proteins of GM and DM ion-exchange chromatography based on SRM data are displayed in the chromatograms in Fig. 4 and in Suppl. Figs. 1 and 2. Comparison of the DM with the GM revealed that although both modes were performed with the same chromatographic AEX material, significantly different elution profiles were obtained.

3.3.1. Comparison of the selectivities of AEX chromatography in gradient mode versus displacement mode

One of the main factors which make it possible to use two separation techniques as orthogonally is that compounds are separated with different selectivities (different orders of elution) by each individual method. The different selectivities of DM and GM is considerably evident in Fig. 4. Since in the chromatograms in Figs. 3, 4 and Suppl. Figs. 1, 2 the retention times are normalized a difference in retention time is equivalent with a difference in the elution order. In DM coeluting serpina 1 (SERPINA1) is well separated from most of the other proteins. In GM SERPINA1 coelutes with Albumin. Further good examples for different elution orders are apolipoprotein AI (APOAI), SH2-Glycoprotein (ASGH) and transferrin (TF) can be pointed out. APOAI elutes in DM with maximum intensity late in fraction 27 but in GM a much earlier elution can be observed. This same behavior is displayed by SH2-Glycoprotein (ASGH), which elutes in fraction 26 in DM and in fraction 12 in GM. Interestingly transferrin (TF) shows almost no retention in GM but starts to elute in DM in fraction 13. This same elution profile can be observed for alpha-1-antitrypsin. This co-elution may indicate that these proteins are still in their native folding allowing a direct interaction between these proteins (Suppl. Figs. 1, 2). The overall comparison of obtained elution profiles demonstrates that almost every protein shows a different elution behavior in DM compared to GM (Fig. 4). Thus a combination in a multidimensional fractionation procedure is reasonable which may offer the chance to investigate protein species in much more detail in top down approaches in future [24,25].

3.3.2. Comparison of the resolution of AEX chromatography in gradient mode versus displacement mode

Regarding the estimation of the resolution of the separations the presence of protein species can be used. The occurrence of more than one protein species which are coded by one single gene presumably is responsible for the multiple elution of single

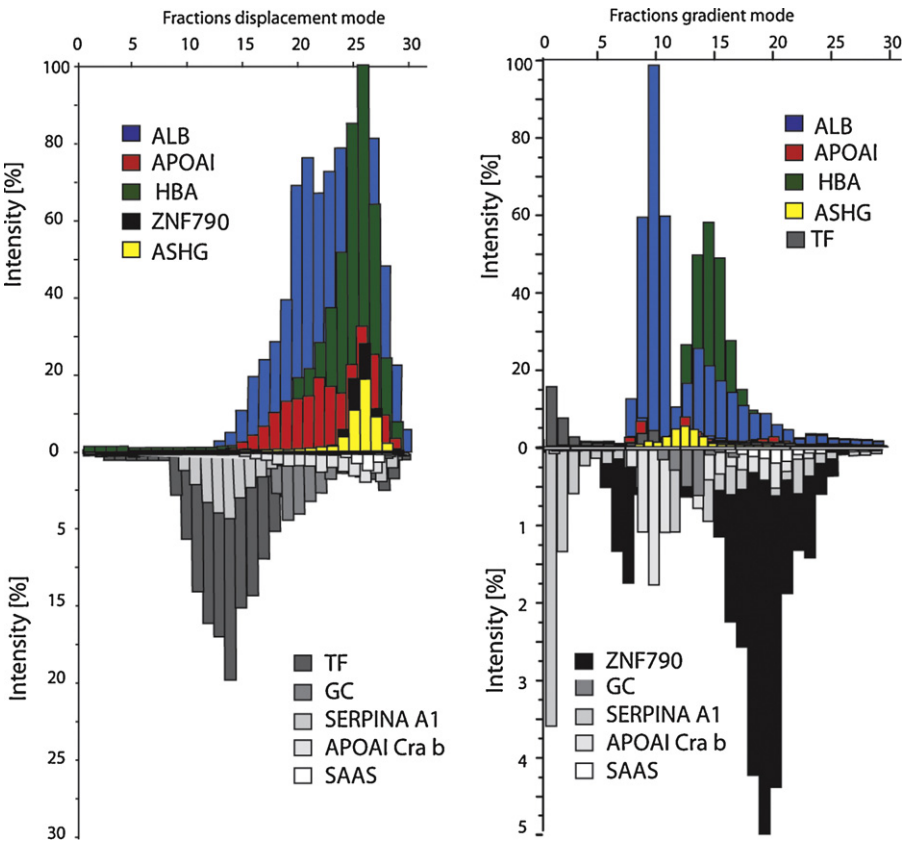

Fig. 4. Protein separation visualization applying selective reaction monitoring (SRM). SRM analysis of the displacement separation. The fraction number is plotted against SRM intensity, here the pooled fractions (60 fractions two per LC/MS run) are analyzed monitoring the transitions of 22 proteins the transitions, 10 of these proteins are displayed for the displacement mode (A) or for GM. (B) The highest SRM response for each mode (intensity) was set to 100% and all other elution profiles were normalized to this profile. To cover the whole intensity range the bottom inlet displays a zoom in to visualize low abundant elution profiles.

proteins (in the sense of coded by single genes), recognizable in Fig. 4 and Suppl. Figs. 1 and 2. This phenomenon is well known from two-dimensional gel electrophoresis of complex protein mixtures where dozens of different protein spots coded by one single gene were identified [26]. The strategy, which we used here for obtaining the elution profiles for 22 selected proteins, is based on the detection of 2 or 3 underivatized tryptic peptides originated from the target protein. However the selected peptides can be present in several protein species. Thus different protein species coded by a single gene with several tryptic peptides in common will not be discriminated by SRM. Nevertheless since these protein species are different in their exact chemical composition the chromatographic behavior can be different. This is obvious in the elution profiles of many of the selected proteins Fig. 4 and Suppl. Figs. 1 and 2. The elution profile of a protein which occurs with several protein species (e.g. APOAI in Fig. 4) than typically shows several maxima. According to the ratio of the number of maxima in the elution profiles of the individual proteins (Suppl. Figs. 1 and 2: DM/GM: Alb 3/2; TF 2/4; APOAI: 2/3; GC 1/4; HBA1,2 1/1; SERPINA1 2/5; SAA4 1/1; ApoAI Cra B 3/3; ZNF 790 1/2; AHSG 1/1) DM has a lower resolution compared to GM. However this is not true for every protein. Alb is better resolved by DM chromatography. Comparison of the elution profiles shown in Suppl. Figs. 1 and 2 again clearly demonstrates the orthogonality of both AEX elution modes.

4. Conclusion

Here we demonstrated by comprehensive qualitative and quantitative mass spectrometry based analysis of proteins in individual AEX chromatography fractions operated in GM versus DM that the latter offers some advantages for separation of complex protein mixtures. With DM a significantly higher number of identified proteins has been yielded. Thus DM is more suitable than GM for investigations of proteomes including top-down proteomics. DM is especially useful for analysis of proteomes if larger amounts of protein extracts are available as this usually is the case according plasma or serum proteins, since the beneficial effects of the DM are most prominent if a larger part of the total binding capacity (50–70%) of the stationary phase is saturated by the sample. DM should not be applied if minute amounts of proteins are available. The orthogonality of the DM and GM may be used for combining both modes in a multidimensional fractionation procedure.

Acknowledgements

This work was supported by the BMBF (Bundesministerium für Bildung und Forschung). Grant: 0315341B.

Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.jchromb.2012.05.037.

References

[1] H. Rasmussen, R.G. Westall, Biochem. J. 67 (1957) 658.
[2] R. Tiselius, A. Kemi, Mineral. Geol. 16A (1943) 1.
[3] R. Ahrends, B. Lichtner, A. Bertsch, O. Kohlbacher, D. Hildebrand, M. Trusch, H. Schluter, J. Chromatogr. A 1217 (2010) 3321.
[4] S. Evans, M. Holstein, S. Cramer, Anal. Chem. 83 (2011) 4184.
[5] J. Newburger, G. Guiochon, J. Chromatogr. 523 (1990) 63.

[6] R. Freitag, C. Horvath, Adv. Biochem. Eng. Biotechnol. 53 (1996) 17.
[7] C. Horvath, A. Nahum, J.H. Frenz, J. Chromatogr. 218 (1981) 365.
[8] F.D. Antia, I. Fellegvari, C. Horvath, Ind. Eng. Chem. Res. 34 (1995) 2796.
[9] J.A. Gerstner, S.M. Cramer, Biotechnol. Progr. 8 (1992) 540.
[10] K.M. Sunasara, R.G. Rupp, S.M. Cramer, Biotechnol. Progr. 17 (2001) 897.
[11] E.J. Cohn, J.L. Oncley, L.E. Strong, W.L. Hughes, S.H. Armstrong, J. Clin. Invest. 23 (1944) 417.
[12] T. Burnouf, Transfus. Med. Rev. 21 (2007) 101.
[13] T. Burnouf, J. Chromatogr. B: Biomed. Appl. 664 (1995) 3.
[14] J.C. Tran, L. Zamdborg, D.R. Ahlf, J.E. Lee, A.D. Catherman, K.R. Durbin, J.D. Tipton, A. Vellaichamy, J.F. Kellie, M. Li, C. Wu, S.M. Sweet, B.P. Early, N. Siuti, R.D. LeDuc, P.D. Compton, P.M. Thomas, N.L. Kelleher, Nature 480 (2011) 254.
[15] P. Jungblut, B. Thiede, U. ZimnyArndt, E.C. Muller, C. Scheler, B. WittmannLiebold, A. Otto, Electrophoresis 17 (1996) 839.
[16] P.R. Jungblut, H.G. Holzhutter, R. Apweiler, H. Schluter, Chem. Cent. J. 2 (2008) 16.
[17] P.R. Jungblut, H. Schluter, Amino Acids 41 (2011) 219.
[18] H. Schluter, R. Apweiler, H.G. Holzhutter, P.R. Jungblut, Chem. Cent. J. 3 (2009) 11.
[19] R.A. Yost, C.G. Enke, Anal. Chem. 51 (1979) 1251.
[20] P. Picotti, B. Bodenmiller, L.N. Mueller, B. Domon, R. Aebersold, Cell 138 (2009) 795.
[21] R. Pieper, Q. Su, C.L. Gatlin, S.T. Huang, N.L. Anderson, S. Steiner, Proteomics 3 (2003) 422.
[22] M. Srajer Gajdosik, J. Clifton, D. Josic, J. Chromatogr. A 1239 (2012) 1.
[23] R. Bischoff, H. Schluter, J. Proteomics 75 (2012) 2275.
[24] A. Armirotti, G. Damonte, Proteomics 10 (2010) 3566.
[25] N.L. Kelleher, H.Y. Lin, G.A. Valaskovic, D.J. Aaserud, E.K. Fridriksson, F.W. McLafferty, J. Am. Chem. Soc. 121 (1999) 806.
[26] J. Klose, C. Nock, M. Herrmann, K. Stuhler, K. Marcus, M. Bluggel, E. Krause, L.C. Schalkwyk, S. Rastan, S.D. Brown, K. Bussow, H. Himmelbauer, H. Lehrach, Nat. Genet. 30 (2002) 385.