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