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High-resolution separations of protein isoforms with liquid chromatography time-of-flight mass spectrometry using polymer monolithic capillary columns

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ABSTRACT

The separation of intact proteins, including protein isoforms arising from various amino-acid modifications, employing a poly(styrene-*co*-divinylbenzene) monolithic capillary column in high-performance liquid chromatography coupled on-line to a time-of-flight mass spectrometer (MS) is described. Using a 250 mm × 0.2 mm monolithic capillary column high-sensitivity separations yielding peak capacities of >600 were achieved with a 2 h linear gradient and formic acid added in the mobile phase as ion-pairing agent. The combination of high-resolution chromatography with high-accuracy MS allowed to distinguish protein isoforms that differ only in their oxidation and biotinylation state allowing the separation between structural isoforms. Finally, the potential to separate proteins isoforms due to glycosylation is discussed.

1. Introduction

The mainstay of the successful mass-spectrometry (MS) based proteomics in the past 15 years is the so-called bottom-up approach to proteomics. It is based on the proteolytic digest of proteins in gel or in solution, followed by chromatographic separation and mass-spectrometric identification of the peptide ions by matrix-assisted laser desorption/ionization-time of flight (MALDI-TOF) or electrospray ionization (ESI) MS [1,2]. The introduction of high-resolution mass spectrometers and of new fragmentation techniques has resulted in the increased interest in top-down approaches to proteomics [3,4]. MS-based top-down proteomics is based on the chromatographic separation of intact proteins followed by their fragmentation in the gas phase using collision-induced dissociation (CID) [5,6], electron-capture dissociation (ECD) [7,8] or electron-transfer dissociation (ETD) [9,10].

Top-down approaches are attractive, because they have the potential to supply information about the modifications of the analyzed proteins, resolving isoforms that are frequently unresolved in bottom-up proteomics experiments, including isoforms resulting from proteolytic processing, oxidative damage, glycosylation, or other forms of post-translational processing. The two main prerequisites for successful top-down proteomics are high-resolution chromatography and high sensitivity and high-resolution mass spectrometry with fragmentation possibilities. To achieve their full potential, top-down workflows have to overcome several challenges: (1) chromatographic separation of complex mixtures of the intact proteins, (2) fragmentation of the proteins in the gas-phase, (3) sensitivity of detection for the intact proteins, and (4) data analysis of the complex MS/MS spectra.

For the separation of intact proteins, reversed-phase liquid chromatography (RPLC) has often been employed, due to excellent solvent compatibility with electrospray interfacing prior to MS detection. However, low protein recovery has frequently been a problem associated with this approach [11]. Polymer monolithic stationary phases have shown great potential for RPLC separations of large biomolecules, including intact proteins [12–15], oligonucleotides [16,17], and peptides [18–20]. Conceptually, this material is very well suited to perform large-molecule gradient-separations since mass transfer is mainly driven by convection, rather than by diffusion due to the absence of mesopores (stagnant zones in microglobules) [21]. In addition, Kelleher et al. demonstrated that polymeric stationary phases led to superior sensitivity over silica-based media in reversed-phase nanocapillary LC, with detection of proteins >50 kDa [22].

Polymer monoliths from styrene and divinylbenzene were initially developed by Svec and coworkers in large I.D. column formats for the separation of styrene oligomers and polymers based on precipitation–redissolution chromatography [23]. Similar materials have more recently been used for the extracts of intact

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membrane proteins containing antenna or/and core proteins [24]. RPLC–MS was performed by applying linear 20–40 min acetonitrile gradients containing 0.05% trifluoroacetic acid and identification was based on comparison of the measured intact mass of the protein and the theoretical relative molecular weight (M_r) values. To achieve full baseline separation between 12 core proteins present in spinach, column temperatures up to 78 °C were applied. A disadvantage of applying higher temperatures is that proteins may be prone to degradation, *i.e.* intra-molecular disulfide bonds may be broken and the amide backbone of the proteins may be hydrolyzed, making protein identification based on the comparison of the experimentally determined and theoretical molecular weight difficult [25]. This separation example shows the need for monolithic columns with improved morphology and the development of longer monolithic columns providing better separation performance at mild LC conditions. Recently, we demonstrated the use of a 50 mm long poly(styrene-*co*-divinylbenzene) monolithic 1 mm I.D. column for the separation of intact proteins [26]. When applying a 1 min gradient peak widths at half height of only 1 s were achieved. At longer gradient duration (120 min) a maximum peak capacity of 475 was observed [26]. Using a 50 mm long capillary poly(styrene-*co*-divinylbenzene) monolith coupled to a LTQ Orbitrap XL mass spectrometer a limit of detection in the low femtomol range was achieved using a standard mixture of nine proteins with a molecular weight ranging between 5.7 and 150 kDa [27]. Using the developed LC–MS method, the 70% ethanol-soluble subproteome of wheat grains was analyzed and 53 different protein masses were obtained from 26 extracted mass spectra. However, it was concluded that identification by comparison of measured molecular masses with masses derived from the published sequences was not possible due to the high sequence homology of gliadins and high number of protein and gene sequences available in the databases. In an experimental study to investigate the effect of column parameters (morphology and length) and gradient time on the performance of capillary poly(styrene-*co*-divinylbenzene) monoliths, it was shown that when using long (250 mm) monolithic columns with optimized morphology a peak capacity of 620 could be achieved for the separation of intact proteins applying a 120 min gradient and UV detection [28].

The present paper discusses the potential of long poly(styrene-*co*-divinylbenzene) monolithic capillary columns for the gradient-elution LC–TOF-MS analysis of intact proteins, including protein isoforms. We address two aspects relevant to the top-down proteomics workflow, namely chromatographic separation at high resolution and MS sensitivity in the detection of the separated proteins. Experimental conditions, including ion-pairing agent (TFA *versus* FA) and gradient time, were adjusted and their effects on retention, peak widths, and signal-to-noise ratios are discussed. We demonstrate the high-resolution separation of a standard 48 protein mixture with low (sub-pmol) protein amounts. In addition, the potential of this set-up for the separation of protein isoforms arising from amino acid modifications is investigated, including oxidation and glycosylation.

2. Experimental

2.1. Chemicals and materials

Acetonitrile (ACN, HPLC supra-gradient quality), formic acid (FA, LC–MS quality), and trifluoroacetic acid (TFA, ReagentPlus quality) were purchased from Sigma–Aldrich (Dorset, United Kingdom). A "Universal Proteomics Standard 1" set was purchased from Sigma–Aldrich (Bornem, Belgium). This set was developed in collaboration with the Association of Biomolecular Resource Facilities (ABRF) Proteomics Standards Research Group (sPRG) (Maryland, USA) and is comprised of one vial containing 48 human source or human sequence recombinant proteins. The total protein content in the vial is 6 mg, which constitutes 5 pmol of each HPLC purified protein. Water was purified in-house using a Millipore Simplicity System (Millipore, Bedford, MA, USA).

A 5 mm × 0.2 mm PepSwift trap column and a 250 mm × 0.2 mm PepSwift RP monolithic columns were provided by Dionex Benelux (Amsterdam, The Netherlands).

2.2. Instrumentation and LC–MS conditions

LC–MS experiments were conducted using an UltiMate 3000 Proteomics MDLC system (Dionex Corporation, Germering, Germany) consisting of a dual-ternary gradient pump with membrane degasser, a thermostatted flow-manager module, and a well-plate autosampler, which was coupled on-line with a maXis ultra-high-resolution qTOF mass spectrometer with a nano-electrospray interface (Bruker Daltonics, Bremen, Germany) equipped with a Proxeon steel emitter.

The separations were performed using the "pre-concentration injection" set-up, with the trap column maintained outside the column oven and the monolithic separation column thermostatted in the oven at 60 °C. After partial-loop injection of 750 or 200 fmol/protein of the ABRF protein mixture, dissolved in water containing 0.02% FA, the sample was desalted on the trap column for 2 min at a flow rate of 20 μL/min and thereafter separated on the 250 mm long monolithic column applying an aqueous acetonitrile gradient from 8% B to 55% B (80% ACN) containing 0.02% FA or TFA at a flow rate of 1.5 μL/min. The chromatographic peak capacity (n_c) was determined applying Eq. (1), where t_G is the gradient time, and W the average 4 sigma peak width:

$$n_c = \frac{t_G}{W} + 1 \quad (1)$$

The data acquisition software was DataAnalysis software 4.0, SP1 (Bruker Daltonics). Spectra were obtained in positive ion mode using the following instrumental parameters: capillary voltage, 4.50 kV; nebulizer, 0.6 bar; dry gas rate, 4.0 L/min; dry gas temperature, 180 °C; funnel RF, 400 Vpp; multipole RF, 400 Vpp; ion energy, 5.0 eV; collision energy, 10.0 eV; collision RF, 1400 Vpp; ion cooler RF, 400 Vpp.

Extracted ion chromatograms (EICs) refer to chromatograms showing the trace of a specific mass (±0.5 Da), *e.g.* 1067.94 ± 0.5. EICs are calculated by summing up the intensities of all specified masses in the mass spectra. Spectra of multiply charged ions were interpreted using the deconvolution algorithms implemented in the instrument software package (DataAnalysis software Version 4, SP2, Bruker).

3. Results and discussion

3.1. Optimization of LC–MS conditions

The effect of ion-pairing agent on the chromatographic performance (retention time, peak width) and mass-spectrometric sensitivity of the gradient-elution LC–TOF-MS separation of intact proteins on the 0.2 mm × 250 mm monolithic capillary column is shown in Fig. 1. To compare the retention properties, both the gradient duration and gradient slope were kept constant throughout the experiments. Retention time, peak width at half height ($w_{1/2}$), and signal-to-noise (S/N) ratios were determined from extracted-ion chromatograms of proteins that elute evenly distributed over the 60 min gradient, see Table 1. No difference in selectivity was observed for the selected proteins. An increase in retention was observed when using 0.02% formic acid FA as ion-pairing agent in the mobile phase, as opposed to 0.02% trifluoroacetic acid (TFA).