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Ultra-High Pressure Fast Size Exclusion Chromatography for Top-Down Proteomics

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Abstract

Top-down mass spectrometry (MS)-based proteomics has gained a solid growth over the past few years but still faces significant challenges in the liquid chromatographic separation of intact proteins. In top-down proteomics, it is essential to separate the high mass proteins from the low mass species due to the exponential decay in S/N as a function of increasing molecular mass. Size exclusion chromatography (SEC) is a favored liquid chromatography method for size-based separation of proteins but suffers from notoriously low resolution and detrimental dilution. Herein we reported the use of ultra-high pressure (UHP) SEC for rapid and high-resolution separation of intact proteins for top-down proteomics. Fast separation of intact proteins (6 – 669 kDa) was achieved in less than 7 min with high-resolution and high efficiency. More importantly, we have shown that this UHP-SEC provides high-resolution separation of intact proteins using a MS-friendly volatile solvent system, allowing the direct top-down MS analysis of SEC eluted proteins without an additional desalting step. Taken together, we have demonstrated that UHP-SEC is an attractive LC strategy for the size-separation of proteins with great potential for top-down proteomics.

Keywords

Protein separation; liquid chromatography; mass spectrometry; UPLC; post-translational modifications

Top-down mass spectrometry (MS)-based proteomics has gained a solid growth over the past few years [1–4]. In contrast to bottom-up MS, the “top-down” MS approach analyzes whole proteins, which greatly reduces the mixture complexity, as no proteolytic digestion is required. More importantly, top-down MS has unique advantages in assessment of post-translational modifications (PTMs) and sequence variants, providing a “bird’s eye” view of all types of modifications without a *priori* knowledge [1–7]. Nonetheless, the full potential of the top-down approach has not yet to be realized in modern proteomics, partly due to the challenges in the separation of intact proteins. Currently, liquid chromatographic (LC) technologies for intact protein separation are severely under-developed [8].

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Conflict of Interests Disclosure

None.

In top-down proteomics, it is essential to separate the high mass proteins from the low mass species due to the exponential decay in S/N as a function of increasing molecular mass resulted mainly from isotopes and charges [9]. Size exclusion chromatography (SEC) is a favored LC approach for the separation of proteins based on sizes or hydrodynamic volumes [8, 10]. SEC has many advantages for separation of proteins including but not limited to simple operating principles, high tolerance of various solvent solutions, preservation of biological activity of proteins, and minimal sample loss because solutes should have negligible interaction with the packing surface in SEC [10–11]. However conventional SEC methods suffer from notoriously low resolution and detrimental dilution as fractions are recovered over a relatively long LC analysis, which significantly limits the use of SEC for protein separation in modern proteomics [8].

In this work, we demonstrated the use of ultra-high pressure (UHP)-SEC for rapid and high-resolution separation of intact proteins for top-down proteomics. The recent development of UHP-LC significantly reduces the analysis time without sacrificing resolution since it allows the use of sub-2 μm small packing particles which minimizes eddy diffusion and mass-transfer resistance in the mobile phase [12]. Moreover, the recently developed BEH organic/inorganic hybrid materials exhibit significantly improved resolution for SEC separation along with mechanical and chemical stabilities comparing to silica-based packing materials [13–15].

All the chromatographic work was carried on an ACQUITY UPLC system with BEH 125 and 200 columns (4.6 mm i.d. $\times$ 150 mm) packed with ACQUITY 1.7 μm BEH particles with mean pore size of 125 $\text{\AA}$ and 200 $\text{\AA}$ (Waters, Milford, USA). We initially used phosphate buffer containing certain amounts of salt to evaluate the performance of UHP-SEC separation of intact proteins since phosphate buffer is a typical mobile phase for SEC separation. Six standard proteins with a molecular mass ranging from 669 kDa to 6.5 kDa were injected individually or in a mixture into BEH125 column (Figure 1). All proteins were eluted in 4 min at 0.4 mL/min flow rate with good peak shape and high efficiency. Intact proteins of BSA (66.4 kDa), ovalbumin (Ova, 44.3 kDa), cytochrome C (Cyt, 12.4 kDa), and aprotinin (Apr, 6.5 kDa) were baseline separated, whereas thyroglobulin (ThG, 669 kDa) and immunoglobulin G (IgG, 150 kDa) were only partially separated. This is consistent with the product specification provided by Waters that BEH125 column is designed for the separation of peptides and proteins in the MW range of 1–80 kDa [15]. In contrast, another UHP-SEC column, BEH200, is designed to characterize proteins in mass range of 10–450 kDa. Indeed BEH200 exhibited much better separation for larger proteins such as ThG and IgG than BEH125 (Supplementary Figure 1). All peaks eluted in 5 min from this BEH200 column at 0.4 mL/min flow rate, which was slightly longer than that observed for BEH125. The peaks were slightly broader in the LC spectrum by BEH200 (Supplementary Figure 1A) in comparison to BEH125 (Supplementary Figure 1B). Good separation reproducibility was achieved with the RSD of elution time less than 0.5% (data not shown).

Next we evaluated the effect of salt concentration on the separation of proteins in UHP-SEC with the goal to minimize the salt content in the mobile phase (Supplementary Figure 2). We found that the decrease of salt concentration from 200 mM to 20 mM NaCl in 50 mM monosodium phosphate (NaH_2PO_4) aqueous buffer did not significantly alter the protein separation profiles. The peak shapes and elution times of large proteins such as ThG, IgG, BSA and Ova did not change appreciably whereas only minor changes were observed for smaller size proteins such as Cyt and Apr. To further reduce the salt content, the minimum concentration of NaH_2PO_4 mobile phase was investigated. We found that 50 mM phosphate buffer without addition of salt (i.e. NaCl) was the minimum concentration to achieve

sufficient ionic strength to minimize the non-ideal secondary interactions [11] for satisfactory SEC separation.

Since sodium phosphate mobile phase is not MS-friendly, we then sought to use a MS-friendly volatile solvent, ammonium acetate (NH₄AC), for SEC separation of proteins (Figure 2A). Fortunately, intact proteins were separated in 50 mM NH₄AC mobile phase with comparable resolution and retention time as those observed in 50 mM NaH₂PO₄ solvent system (Supplementary Figure 3). All proteins were eluted in less than 7 min at a flow rate of 0.2 mL/min. Only slightly longer elution times for smaller size proteins, Cyt and Apr, were observed in NH₄AC solvent in comparison to NaH₂PO₄. Fractions in MS-friendly NH₄AC solvent were collected and directly injected into LTQ/FT for both low and high-resolution MS analysis without an additional desalting step (Figure 2B and C). The broad-band low resolution mass spectra (Figure 2B) clearly demonstrated a single protein component in each fraction, confirming the effective separation of proteins by UHP-SEC. High-resolution MS provided accurate molecular weight (MW) measurement for these proteins accordingly. The measured MW of BSA is 66426.44, which matches the calculated MW of 66426.70 based on the sequence provided in the UniProtKB/Swiss-prot database (P02769) with the consideration of the formation of 17 disulfide bonds. The measured MW of Cyt is 12357.32, matching a calculated MW of 12357.33 with a mass discrepancy of 0.8 ppm based on the DNA-predicted sequence (UniProtKB/Swiss-prot P00004) after considering modifications including removal of methionine, formation of acetylation, and a covalently bonded heme group. For Apr, the measured MW is 6511.08, which matches with the calculated MW of 6511.05 (4.6 ppm) based on the DNA-predicted sequence (UniProtKB/Swiss-prot P00947). A complex modification profile was observed for Ova (data not shown) presumably due to glycosylation and phosphorylation (shown in Figure 2C is the most abundant protein modified form).

Furthermore, the proteins collected from SEC fractions were analyzed by tandem MS (MS/MS) to characterize the sequence and modifications. Figure 3A showed the representative collisionally activated dissociation (CAD) spectrum of Cyt. One CAD produced 19 b ions and 53 y ions with 10 complementary ion pairs (Figure 3B), which unambiguously confirmed the sequence of Cyt and modifications on the N-terminal including the removal of N-terminal methionine and acetylation at the new N-terminus as well as the covalently bonded heme group. This represents a typical example of top-down MS analysis of intact proteins separated by UHP-SEC.

We have reported here that UHP-SEC with BEH columns exhibited significantly enhanced resolving power for fast SEC separation of intact protein in a wide MW range. This is consistent with previous applications by Wheat et al. that UHP-SEC can provide separation of intact proteins within a very short and similar time frame [14–15]. The effect of pore size on resolution and peak shapes of a typical mixture of intact proteins separated on UHP-SEC columns has also been investigated [15]. More importantly, here we demonstrate that such a high-speed UHP-SEC analysis showed great potential for top-down proteomics. Conventional SEC analysis requires at least 30 min to hours which can cause detrimental dilution [16]. It is therefore desirable to reduce the analysis time without sacrificing resolution, especially for high throughput 2DLC analysis. Different approaches have been evaluated to reduce the SEC analysis time, including reducing the column length, increasing the flow rate, and decreasing the particle size of the column stationary phase [16]. The most attractive approach to reach the goal is to use small packing particles (<2 μm) which minimize eddy diffusion and mass-transfer resistance in the mobile phase [16]. Indeed, we achieved fast separation of intact proteins (6–669 kDa) using a UPLC system with the BEH columns packed with 1.7 μm organic/inorganic hybrid particles, as well as detailed sequence characterization of the intact proteins by top-down MS.

More importantly, we have demonstrated that this UHP-SEC could provide high-resolution separation of intact proteins using a MS-friendly solvent system. Recently, Tran and Doucette invented a gel-eluted liquid fraction entrapment electrophoresis (GELFrEE), which provides broad mass range protein separation in less than 90 min [17]. Kelleher group has successfully coupled GELFrEE with RPLC and demonstrated its use for top-down proteomics [2]. Although GELFrEE system is very effective for intact protein separation, it uses non-MS compatible detergent (i.e. SDS), which requires extra steps to remove the detergent before MS analysis. Here we have shown that UHP-SEC is an attractive MS-friendly LC strategy for the size-separation of proteins in top-down proteomics.

We expect UHP-SEC could either be utilized as the initial prefractionation step or be coupled with RPLC and ion exchange chromatography as a multi-dimensional LC separation strategy for intact protein profiling from complex mixtures in top-down proteomics. The current limitation of UHP-SEC is the relatively large column diameter since BEH columns are only available in analytical column size that consumes much more samples than typically used in a proteomic study. Further development of a capillary version of the BEH columns with nanoUPLC is needed to fully realize the potential of UHP-SEC for top-down proteomics.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Abbreviations

LC	liquid chromatography
MS	mass spectrometry
PTMs	post-translational modifications
UHP	ultra-high pressure
SEC	size exclusion chromatography
MW	molecular weight
ThG	thyroglobulin
BSA	bovine serum albumin
IgG	immunoglobulin G
Ova	ovalbumin
Cyt	cytochrome C
Apr	aprotinin
CAD	collisionally activated dissociation
GELFrEE	gel-eluted liquid fraction entrapment electrophoresis

2DLC

two-dimensional liquid chromatography

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Figure 1. UHP-SEC separation of standard proteins

Proteins were injected individually in (A) and injected in a mixture in (B). Conditions: BEH125 column, 4.6 mm i.d. $\times$ 150 mm; mobile phase, 0.2 M NaCl in 50 mM NaH_2PO_4 at pH 4.5; flow rate, 0.4 mL/min; column temperature, 40 $^\circ\text{C}$; UV detection, 214 nm. ThG (669 kDa), IgG (150 kDa), BSA (66.4 kDa), Ova (44.3 kDa) Cyt (12.4 kDa), Apr (6.5 kDa).

Figure 2. UHP-SEC separation and MS analysis of intact proteins in a MS-friendly solution
 (A) SEC separation of a standard protein mixture. Conditions, BEH125 column, 4.6 mm i.d. $\times$ 150 mm; mobile phase, 50 mM NH_4AC solution at pH 4.5; flow rate, 0.2 mL/min; column temperature, 40 $^\circ\text{C}$; UV detection, 214 nm. Protein fractions were collected and analyzed directly on low-resolution LTQ (B), and high-resolution LTQ/FT (C) MS. The measured masses are the most abundant masses. The denoted peak in A (*) is presumably BSA aggregates.

Figure 3. Representative top-down MS/MS analysis of proteins separated by UHP-SEC
 One CAD spectrum of Cyt from isolated precursor ions (M^{12+}) in (A); and its fragmentation map in (B). The fragments were assigned based on the protein sequence of equine cytochrome C obtained from the Swiss-Prot protein knowledgebase (P00004) with the removal of N-terminal methionine and the new N-terminal acetylation as well as a covalently bonded heme group.