

Two-Dimensional SEC/RP Capillary LC for Top-Down Proteomics Analysis

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Introduction

Top-down proteomic liquid chromatography (LC) combined with mass spectrometry (MS) is used to investigate protein structure and post-translational modifications (PTM) through liquid-phase separation and MS analysis of intact proteins. The advantage of top-down over bottom-up approaches is the absence of a proteolytic cleavage step. This keeps all protein information within one molecule and does not multiply sample complexity. However, working with intact proteins introduces challenges for the analytical technology used. Proteins are more difficult to separate than peptides using standard LC methods and more difficult to study by MS.

Recently, different electron-based dissociation techniques, e.g., electron-capture detection (ECD) and electron-transfer dissociation (ETD), have been successfully applied to intact proteins to obtain sequence and PTM information. The successful application of these techniques in tandem with MS for protein mixtures requires a separation step. But powerful LC methods for proteins are published infrequently, and need to be developed and optimized for direct interfacing with MS.

This study describes a two-dimensional (2D) capillary-scale LC method for the separation of intact proteins. Proteins are separated and fractionated on a capillary size-exclusion chromatography (SEC) column. Next, proteins contained in the size fractions are separated on reversed-phase (RP) capillary monolithic columns prior to UV and MS detection. The method has been optimized with respect to SEC fractionation and RP gradient conditions for standard proteins and complex mixtures.

Experimental

Capillary-scale SEC and nano monolithic columns were used to ensure compatibility with the limited sample amounts typically used for proteomics. Fractions were collected manually and reinjected onto a preconcentration monolithic setup for desalting, conditions shown below. The fractionation was time based and was chosen based on the peak width from the SEC column and the collected volume that had to be reinjected. The sample used in this study was a protein mixture as described in Table 1.

Table 1. Protein Mixture Used for the Experiments

Protein	Source	MW (kDa)	Concn. ($\mu\text{g}/\mu\text{L}$)	Injection (pmol)
Thyroglobulin	Porcine	304	0.90	0.30
β -Galactosidase	<i>E. coli</i>	135	0.90	0.67
Albumin	Bovine Serum	69.3	0.90	1.30
Glucose Oxidase	<i>Aspergillus Niger</i>	65.6	0.90	1.37
Albumin	Chicken Egg	42.7	0.90	2.11
β -Casein	Bovine Milk	25.1	0.90	3.59
Trypsin Inhibitor, Type II-T	Turkey Egg White	20.0	0.90	4.50
β -Lactoglobulin A	Bovine Milk	18.4	0.90	4.89
α -Lactoalbumin, Type I	Bovine Milk	16.2	0.90	5.56
Ribonuclease A, Type III-A	Bovine Pancreas	13.7	0.90	6.57
Cytochrome C	Bovine Heart	12.3	0.90	7.32

The experiments were performed using a Thermo Scientific Dionex UltiMate™ 3000 RSLCnano Rapid Separation LC (RSLC) nano system equipped with UV detection. The monolithic setup was coupled to an HC HCT Ion Trap MS.

Capillary Size-Exclusion Chromatography

Column: Thermo Scientific MAbPac™ SEC-1, 300 µm i.d. × 600 mm (two 300 mm columns coupled)

Mobile Phase: 50 mM NaHPO₄, pH 6.8, 300 mM NaCl

Flow: 1.5 µL/min

Injection Volume: 100 nL (with internal loop)

Injection Amount: See Table 1

Detection: UV at 280 nm

Fraction Size: 2 min fractionation for 1–12, 4 min for 13 (last fraction)

RP Chromatography

Column: Thermo Scientific PepSwift™ monolithic column, 100 µm i.d. × 250 mm, with Thermo Scientific Dionex nanoViper™ connections

Trap Column: PepSwift monolithic trap column, 200 µm i.d. × 5 mm, with nanoViper connections

Mobile Phases: A) Water, 0.05% TFA
B) 80% Acetonitrile, 0.04% TFA

Loading Solvent: Water, 0.05% TFA

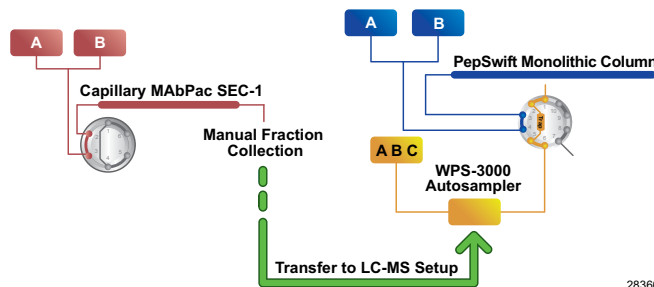
Flow Rate: 1.0 µL/min

Loading Flow Rate: 10 µL/min

Loading Time: 1 min

Gradient: 10–70% B in 15 min, 5 min wash, 10 min equilibration

FIGURE 1. Experimental setup of the SEC/RP top-down workflow.



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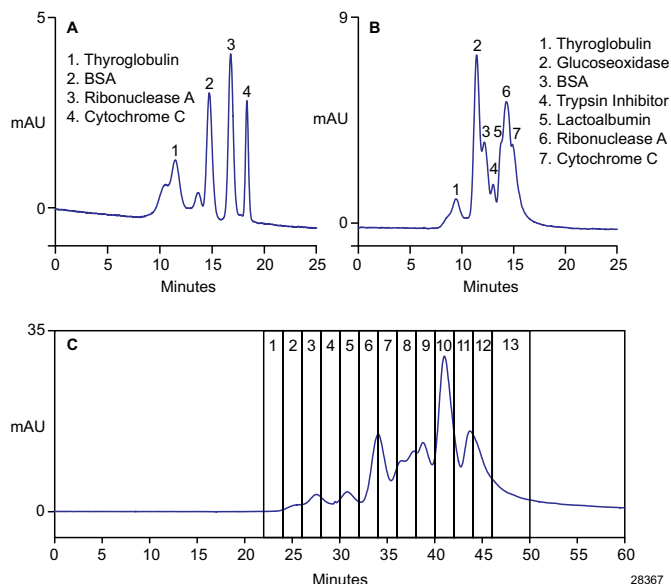
Results

First-Dimension Separation of Intact Proteins—Capillary SEC

SEC is a chromatographic technique that separates species based on their molecular size. There is no interaction between the stationary phase and the molecules; the separation is based solely on the diffusion rate in and out of the pores. The larger proteins have less freedom to move in and out of the pores and, therefore, exit the column faster. The mobile phase is chosen to minimize denaturing. As a result, the MAbPac SEC material under the applied chromatographic conditions separates the proteins in their native state. Because SEC is an isocratic method, there is no focusing on the top of the column. The injection amount should be compatible with the flow rate and column size, and dispersion outside the column should be minimized. Here, a manual injector with internal loop was used to inject 100 nL of sample on the column.

Figure 2 shows the separation of the protein mixture and fractions. With the timed fraction collection, peaks can appear in several fractions, and fractions 6 and 7 are good examples. This method serves as a prefractionation technique to reduce sample complexity. SEC's strongest asset is the minor influence the separation conditions have on the sample integrity.

FIGURE 2. A) Isocratic separation of the 4-protein mixture; the plates per meter found for cytidine are 65,000. B) Isocratic separation of the 7-protein mixture; properties can be found in Table 1. C) UV trace of the separation and fractionation of the 11-protein mixture on two coupled capillary MAbPac SEC-1 columns. The 13 fractions are indicated.



Second-Dimension Separation of SEC Fractions—Nano RP Monolith

The 11-protein mixture was created as a proof of concept sample. The dynamic range in molecular weights (12–305 kDa) was more important than the number of proteins in the sample. The 11-protein mixture was injected on the monolithic column unfractionated and after SEC fractionations. When comparing the one-dimensional (1D) RP analysis with the combined fraction chromatograms (Figure 3), all peaks are visible and all (except the peak at 9 min) correspond roughly in peak intensity. The differences in chromatography are mainly attributed to overloading of the monolithic column, which causes peak broadening and, therefore, a slight change in the elution profile (peaks at 13.5 min).

FIGURE 3. UV trace overlay of unfractionated sample (blue) and fraction injections (green) on the monolithic RP column. The blue trace represents the injection of the unfractionated sample in the same amount as was injected on the SEC column. The green traces are the overlaid fractions from the SEC column. The peak width in the blue traces is broad (PWHH 15 s) due to overloading of the monolithic column.

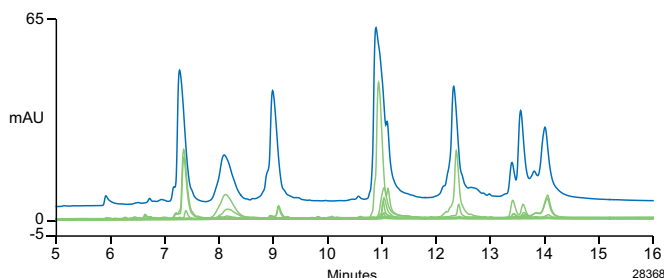
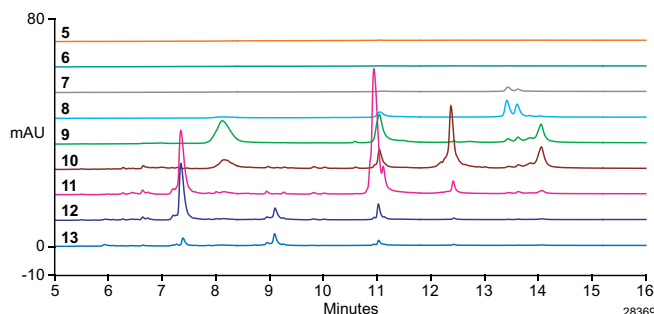


Figure 4 shows the fractions starting at fraction 5 (fractions 1–4 were too low in sample amount to be visible). The occurrence of peaks in consecutive fractions is explained above by the timed fraction collection.

The distribution of peaks over the fractions is interesting. RP chromatography separates according to hydrophobicity, and often it is assumed that larger proteins, by definition, exhibit more hydrophobicity. What the peak distribution shows, most strongly for the component eluting at 9 min, is that hydrophobicity and size are not linearly correlated; smaller proteins can exhibit more hydrophobicity than bigger ones. The native conditions of SEC and denaturing conditions of RP chromatography have an influence on this as well. Orthogonality cannot be determined for such a simple mixture; however, the components are nicely distributed. Optimizing the fraction collection will minimize occurrence of identical peaks in consecutive fractions.

FIGURE 4. UV trace overlay of fraction injection from Figure 2, starting at fraction 5. The numbers in the chromatogram indicate the fractions.



MS Data

Top-down proteomics involves intact protein MS as well, which represents a challenge on its own. For the chosen protein mixture, the theoretical-charge envelopes can be calculated. Table 2 shows what the charge state (n) is for a peak close to 900 m/z (center of the applied mass range) for each protein, and also what the difference in m/z (Δ) is for two neighboring peaks in the charge envelope. From Table 2, it is evident that the resolution required to identify the larger proteins can only be obtained on high-end instruments.

Table 2. Protein MW Charge State Relationship for Detectable Mass Range in Electrospray Ionization

Protein	MW	n for m/z 900	Predicted m/z		Delta
			n	n-1	
Thyroglobulin	305,000	339	900.71	903.37	2.66
β -Galactosidase	135,000	150	901.00	907.04	6.04
Glucoseoxidase	65,600	72	912.11	924.94	12.83
BSA	60,000	66	910.09	924.08	13.99
Albumin	43,000	47	915.89	935.78	19.89
β -Casein	25,100	27	930.63	966.38	35.75
β -Lactoglobulin	18,400	20	921.00	969.42	48.42
Ribonuclease A	17,600	19	927.32	978.78	51.46
Lactoalbumin	16,200	18	901.00	953.94	52.94
Trypsin Inhibitor	14,700	16	919.75	981.00	61.25
Cytochrome C	12,000	13	924.08	1001.00	76.92

Figure 5 shows the MS data for the same fractions as in Figure 4. The intensity differences compared to the UV detection are based on the more selective detection of a mass spectrometer. UV detection is based on the absorption by certain molecular bonds in the peptide backbone. MS detection is a far more complex process influenced by more parameters.

Figures 5B–5E show selected peaks from the fraction analysis. The top two have such condensed charge envelopes that the peaks are identified by their retention time from individual protein injections (data not shown). The first is glucose oxidase (65.6 kDa, 14 min) and the second is chicken albumin (42.7 kDa, 14.5 min). Note that the elution order is in correspondence with SEC, but the smaller of the two shows more hydrophobicity.

The two following components are identified as lactoglobulin (18.4 kDa) and cytochrome C (12.2 kDa) based on MW and retention time from individual protein injections (data not shown).

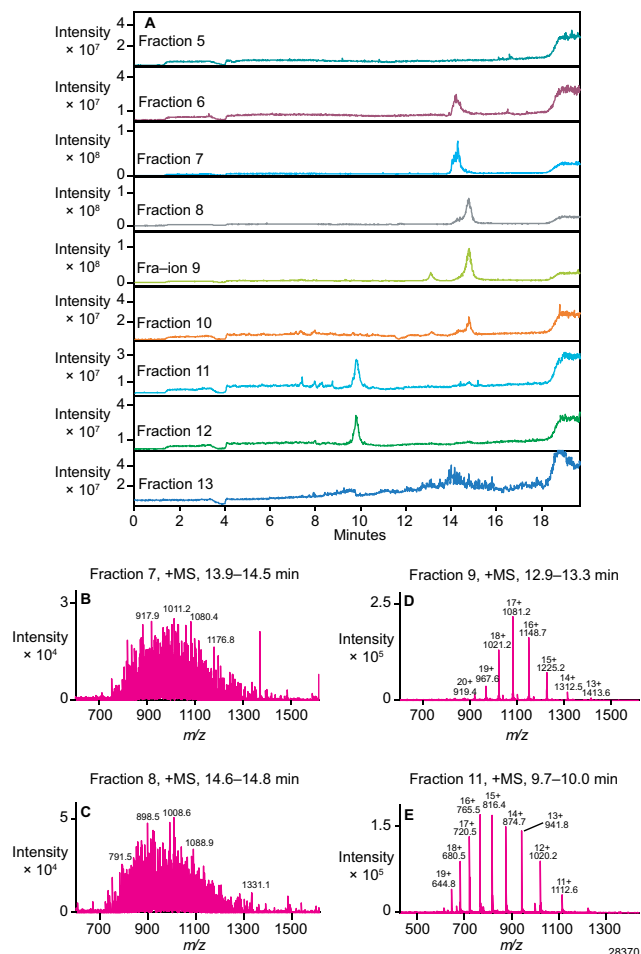
Conclusion

Capillary SEC columns were successfully prepared and allow a size-based separation of intact protein with sample amounts compatible to proteomics.

Capillary SEC can be off-line coupled to monolithic RP nano columns. These columns are ideal for top-down proteomics due to their excellent separation performance for intact proteins over a wide range of molecular weights.

Size and hydrophobicity present complementary separation mechanisms when the minimal influence of the SEC eluents and the resolution and speed of the monolithic column are a good combination.

FIGURE 5. A) Overlay of total ion chromatogram (TIC) data of the fractions from Figure 4. **B-E)** Averaged mass spectra from the fractions and peaks indicated. By mass, retention time, and SEC elution, the proteins were identified as: **B)** glucose oxidase; **C)** chicken albumin; **D)** lactoglobulin; and **E)** cytochrome C.



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