An Optimized Two-Dimensional LC-MS System for Separation/Identification of Ribosomal Proteins

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Introduction

Effective separations and accurate mass or molecular weight (MW) measurements are vital to protein identification and post-translational modification (PTM) determinations. In spite of a number of limitations, 2D gels are still the most predominant technique for complex protein mixture analysis. Approximate MWs can be obtained from 2D gels, and in-gel digest can often yield identifications, but rarely is the information accurate enough to facilitate protein identification and PTM determinations.

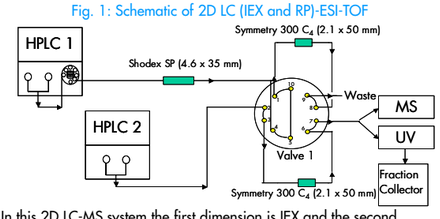
Multi-dimensional chromatography (MDC) is becoming an attractive alternative to 2D gels for intact protein separations. A significant advantage of MDC is its easy coupling to electrospray TOF MS for direct and accurate MS measurement of intact proteins.

In this poster, we present a 2-dimensional chromatographic system coupled to ESI-TOF MS for separation and identification of yeast ribosomal proteins. Ion exchange (IEX) chromatography and reversed-phased (RP) chromatography are employed as the first and the second dimension of the separation, respectively. Accurate masses are then used for tentative protein identifications.

Instrumental Conditions

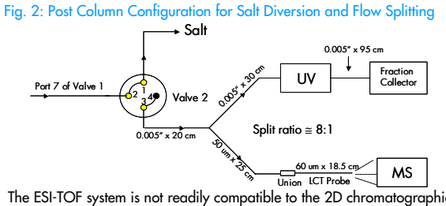
- Sample preparation
  - Mix 50 µl of a ribosomal sample with 5 µl of 1M $MgCl_2$ and 100 µl of glacial acetic acid. Vortex the mixture, centrifuge at 10000 rpm for 2 mins, and collect the supernatant.
  - The final concentration of total protein: ~3 mg/ml.
- Injection volume
  - 5 µl (unless otherwise indicated)
- Ion exchange
  - Column: Shodex® SP 420N (4.6 x 35 mm, non-porous)
  - Eluent A: 50 mM methylamine, 6M urea, 0.5 mM DTT, pH adjusted to 5.60 with acetic acid, 10% (v/v) acetonitrile
  - Eluent B: Eluent A plus 1M NaCl
  - Flow rate: 0.4 ml/min
  - Gradient: step gradient (0-90%B)
- Reversed phase
  - Column: Symmetry300™ C_4, 3.5 µm (2.1 x 50 cm)
  - Eluent A: 0.1% TFA in water
  - Eluent B: 0.1% TFA in acetonitrile
  - Flow rate: 0.5 ml/min
  - Gradient (linear):
    10-60%B in 18 min (1D)
    20-50%B in 18 min (2D)
- ESI-TOF (Micromass® LCT)
  - Source: 3 kV (capillary), 60 V (Sample cone), 3 V (extraction cone), 450 (RF lens)
  - Temperature (°C): 250 (desolvation), 130 (source)
  - Gas flow (L/hr): 100 (cone), 400 (desolvation)
  - MCP detector: 2700 V
  - Max Flight time: 65 µS

Fig. 1: Schematic of 2D LC (IEX and RP)-ESI-TOF



In this 2D LC-MS system the first dimension is IEX and the second dimension is RP (Fig. 1). A step gradient is performed on the IEX column.The two RP columns alternately collect fractions from the IEX column. During each IEX step, collection occurs on one RP column, while the RP separation is carried out on the other RP column.

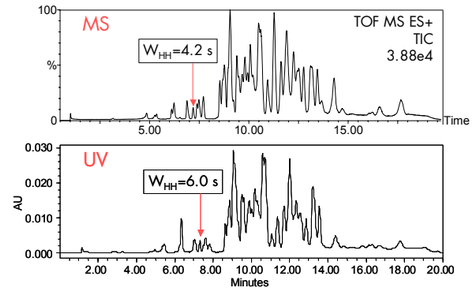
Fig. 2: Post Column Configuration for Salt Diversion and Flow Splitting



The ESI-TOF system is not readily compatible to the 2D chromatographic system unless 1) involatile buffer components from the first dimension (salts and urea) are removed and 2) the flow rate to the MS (0.5 ml/min) is reduced. In the current configuration, Valve 2 is employed as a salt diversion valve, and a splitter is setup to reduce the flow rate (Fig. 2). Involatile buffer components are removed after diverting the mobile phase to waste for several minutes at the beginning of each RP gradient. The flow is split to divert ~11% (55 µl/min) to the MS with the remainder diverted to a fraction collector.

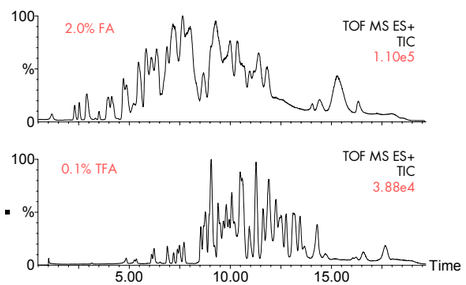
System Optimization

Fig. 3: RP Separation and UV/MS detection of Ribosomal Proteins



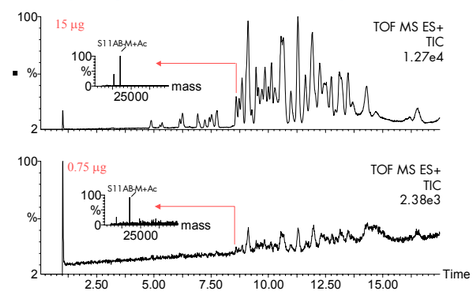
Special attention is paid to the post-column part of the system to avoid significant band-spreading. With the optimized configuration, little band broadening is observed due to the addition of Valve 2 and the splitter. In Fig. 3, the ribosomal proteins are separated and detected with both MS and UV. Peak width measurements at half height (W_{HH}) show that the apparent MS resolution is actually slightly better than that observed by UV.

Fig. 4: 1D RP Separation Comparing TFA and FA



Even though the MS detection sensitivity was better using a mobile phase of water-acetonitrile containing 2.0% formic acid (FA), a mobile phase containing 0.1% TFA was chosen for the 2D system as it provided significantly better resolution, with good sensitivity (Fig. 4).

Fig. 5: High Sensitivity RPLC-MS Analysis Using 0.1% TFA



Effective detection limits for the ribosomal sample were analyzed with RPLC-MS using 0.1% TFA. In Fig. 5, the MS spectra obtained indicate that good MS sensitivity is produced with the TFA-containing mobile phase. Many proteins can still be detected and mass calculated when as little as 0.75 µg of total protein is injected (approximate loading amount for each protein: 0.5 pmol).

2D LC-MS

Fig. 6: 2D Chromatography of Ribosomal Sample

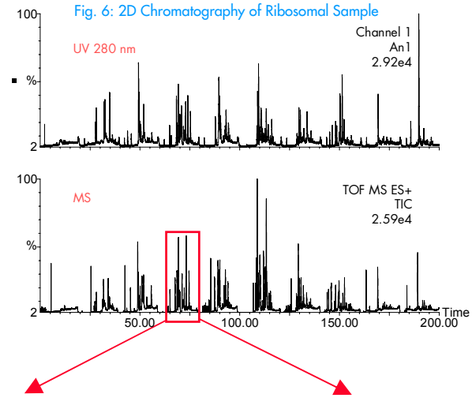


Fig. 7: Expanded TIC for Fraction 3

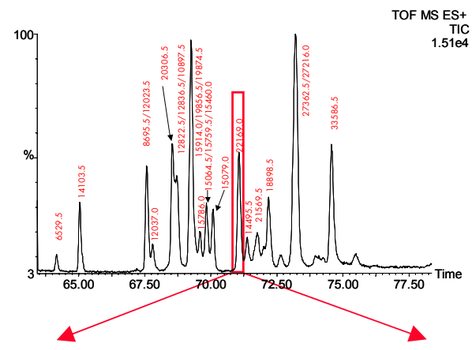
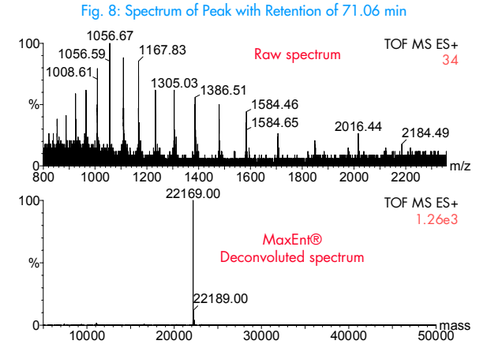


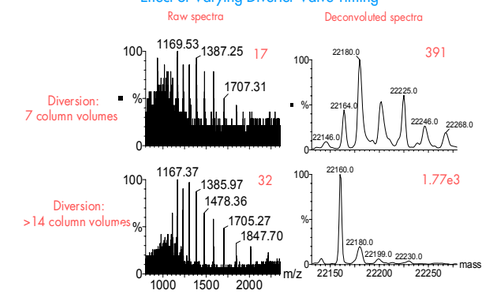
Fig. 8: Spectrum of Peak with Retention of 71.06 min



The ribosomal sample is well separated with the optimized 2D LC system. There are more than 100 peaks found in Fig. 6, most of which have good resolution from each other (e.g. Fig. 7). The raw MS spectra of these peaks were deconvoluted to get the masses of intact proteins (Fig. 8). Since a carefully calibrated LCT system provides accurate MW data (avg. error of deconvoluted MW: ~50 ppm), this can be very useful in identifying the proteins and possible PTMs (Table 1). This 2D separation of intact ribosomes has allowed the tentative identification of >80 unique primary structures with a subunit coverage of ~85%.

2D LC-MS

Fig. 9: Spectra of Peak with Retention of 113.2: Effect of Varying Diverter Valve Timing



Involatile components in IEX solvents (e.g. urea,salts) compromise MS data quality, so it is imperative to divert these to waste. Here, when the valve timing is properly set (diversion volume > 14 column volumes), there are few adducts observed. However, when the diversion is only 7 column volumes, there are numerous adducts observed (Fig. 9). Thus the resulting spectrum is difficult to interpret as the peak with the highest apparent signal is actually an adduct peak.

Table 1: Identification of Selected Ribosomal Protein Peaks from Fraction 3

Retention Time (min)	Observed mass	Assignment	Calculated MW
65.04	14103.5	L26A-M	14102.6
67.57	12023.5	L33A-M	12023.0
67.57	8695.5	L38-M	8695.4
67.80	12037.0	L33B-M	12037.0
68.71	12822.5	L31A-M	12822.0
68.71	12836.5	L31B-M	12836.0
69.25	19874.5	L6A-M+Ac	19872.5
69.25	19856.5	L6B-M	19855.4
69.25	15914.0	S15-M+Ac	15912.8
69.58	15786.0	S19A-M	15786.0
71.06	22169.0	S9B-M	22167.6
71.75	21569.5	L9A or L9B-M+Ac	21569.2 or 21568.1
74.57	33586.5	L5-M	33583.9

Most proteins observed can be tentatively identified based on their expected MWs (Table 1). However, some proteins can not be identified with the MW information alone as there may be other proteins with similar MWs. Definitive identification can be realized by combining the data obtained here with peptide fingerprints or MS/MS data obtained from the portion of sample diverted to the fraction collector.

Conclusion

- An optimized 2D LC-MS system has been set up for intact ribosomal protein analyses.
  - Post-column diversion valve and splitter setup permits on-line LC/MS with fraction collection.
  - Minimal band-spreading is observed due to the post-column configuration.
  - A TFA containing mobile phase used for the second dimension provides excellent resolution with sub pmol MS sensitivity.
- More than 100 protein peaks are resolved with the 2D system.
- Tentative protein identifications (~85% of known ribosomal subunits) can be readily performed based on intact mass information.

Acknowledgement

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