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Characterization of Noncovalent Protein Complexes by LC and Multidimensional LC Coupled with Online ESI-Tof MS Detection

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Overview

- Noncovalent protein complexes (NPC's) are the functional biological unit for many cellular activities.
- Thus, characterization of NPC's has become a major research effort in the post-genomic era.
- Ionization and detection of weakly associated NPC's is a challenging task, since a gentle transition of the complex from solution to the gas phase requires significant attention during methods development.
- Optimized electrospray conditions permit direct detection of intact NPC's, or its constituent subunits.
- Online separations can be coupled to ESI-MS to derive additional insights about complex structure and composition.

Introduction

The desire to describe the molecular machinery within the cell has led researchers to systematically characterize NPC's. Mass analysis of the intact complex is useful for discerning subunit stoichiometry and ligand interactions, while protein and peptide level analysis is more effective at characterizing and localizing subunit modifications. The gas phase ionization and detection of NPC's by MS methods is a challenging task since the transition of the complex between the solution and gas phase requires effective desolvation without disrupting complex structure (1,2). Furthermore, NPC analysis can be coupled with online separations to concentrate samples or separate complicated mixtures (3). We have developed a methodology to detect and characterize a tetrameric yeast alcohol dehydrogenase (ADH) complex using online SEC/MS analysis, while analyzing constituent subunits by a 2-D "heartcut" SEC/RP/MS methodology.

Yeast ADH forms a homotetrameric complex where each monomer (~37kD) is liganded to two divalent zinc ions. One zinc is essential for the activity and other is required to maintain structural integrity. Thus, the tetrameric complex can be expected to contain eight zinc ions for an intact complex mass of 147505 Da.

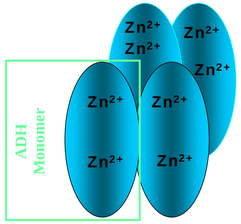

Figure1: Yeast Alcohol dehydrogenase (ADH) structure (Tetramer)

Methods

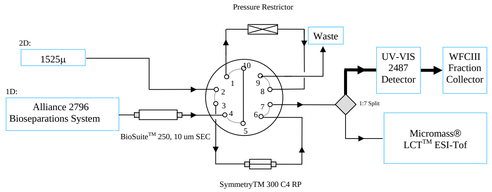

Figure 2: Schematic of the integrated Alliance® 2D Bioseparations System (Waters). During SEC/MS analysis of the intact complex, SEC column effluent was directed to the UV detector and ESI-Tof MS source through a passive flow splitter. During SEC/RP/MS a 10-port 2-position valve permitted direction of the flow to waste though a backpressure restrictor, or through a reversed phase column. Following transfer of the peak, the RP flow through the valve was directed to the UV detector and MS source through the flow splitter.

Sample Preparation:
RP/MS: 9 µg yeast ADH was resuspended in 0.35% formic acid.
SEC-MS and SEC/RP/MS: Yeast ADH (18 µg) was resuspended in 20 mM ammonium acetate buffer, pH 5.5.

SEC / Heartcut 2D LC (SEC-RP) / RP Conditions:
LC System: Waters Alliance® 2D Bioseparations System

SEC Conditions: Waters BioSuite™ 250,10 um (7.5 x 300 mm), 20 mM ammonium acetate, pH 5.5 (0.25 ml/min).

RP Conditions: Waters Symmetry™ 300, C_4 (2.1 x 50 mm), 45 °C, linear gradient 5-80% of ACN in 0.35% formic acid (0.5 ml/min).

Mass Spectrometry Conditions (adapted from Ref. 4,5):

MS System	Waters Micromass® LCT™ ESI-Tof
Ion Mode	Positive
ESI source voltage	3 kV
Cone Voltage	140 V (1D SEC/MS), 45 V (2D SEC/RP/MS & RP/MS)
Desolvation/Source Temp	200 °C /110 °C
Post source pressure	7.1mbar (SEC-MS), 1.7 mbar (SEC/RP/MS)
Data Acquisition	500-10000 (m/z)
Calibrant	2 mg/ml cesium iodide in 50 % IPA
Software:	Waters MassLynx™ 4.0 & MaxEnt 1

Results and Discussion

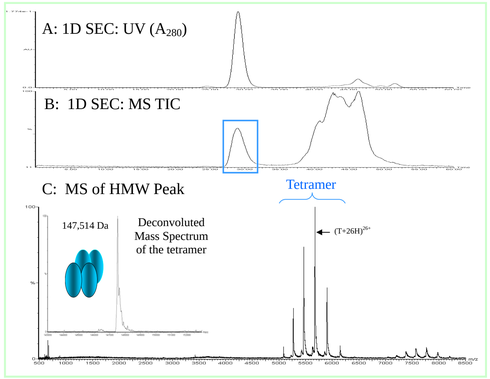

Figure 3: Analysis of the tetrameric yADH complex by online SEC/ESI-Tof-MS/UV. The UV chromatogram **(A)** shows a single high MW peak, while the MS TIC **(B)** shows this peak and significant signal from lower MW components. The mass spectrum **(C)** and MaxEnt1 deconvoluted spectrum (inset) were obtained by summing spectra under the high MW SEC peak. The mass observed for the tetramer (147,514 Da) is within ~50 ppm of that predicted mass.

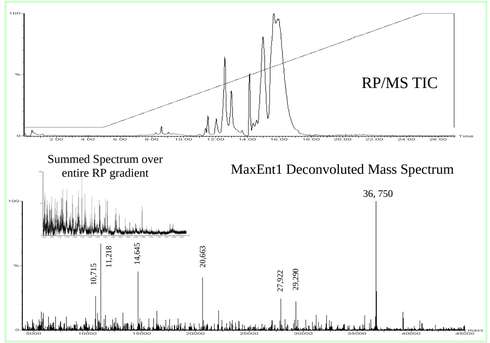

Figure 4: RP/MS of the ADH complex. The TIC **(Top)** from the RP/MS analysis of yADH shows a high degree of complexity. If we sum spectra for the entire gradient **(Bottom, inset),** we produce a deconvoluted spectrum which reveals the presumptive 37kD yADH monomer, as well as a large number of lower mass species.

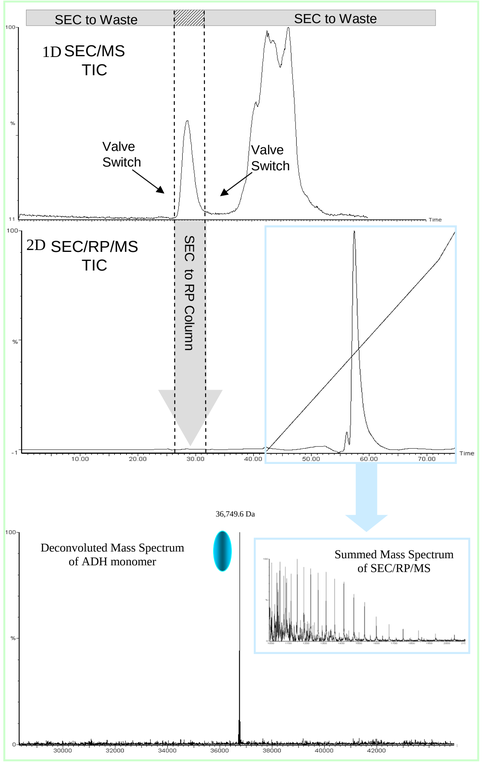

Figure 5: Characterization of ADH subunits by online "heartcut" 2D LC (SEC/RP)/ESI-Tof-MS. Combining SEC with the RP separation into an online 2D "heartcut" separation permits us to selectively transfer the ADH complex to the 2nd dimension for RP analysis. Thus, everything we observe in the 2D SEC/RP/MS run is directly associated with the complex observed during SEC/MS analysis.

Timing for the valve switches at the beginning and end of the SEC peak was derived from the initial 1D SEC/MS separation (**Top**), and produced a single RP/MS peak (**Middle**), that revealed only the expected monomeric ADH subunit (**Bottom**). The observed subunit mass is ~57 Da larger than predicted from the sequence, indicating a possible protein modification or sequence variation.

Conclusions

- The combination of SEC/MS and "heartcut" 2D SEC/RP/MS experiments provided a fast and efficient approach for characterizing an ADH noncovalent protein complex, and it's constituent subunits.
- Online SEC-MS showed the tetrameric structure of the complex, while the heartcut 2D-MS analysis identified the subunits as homogeneous 37 kD components.
- Reversed phase analysis of the ADH complex was insufficient to describe the subunit structure, as low molecular weight contaminants were also present in the sample.
- The use of the SEC/RP heartcut separation permitted direct analysis of only those proteins present within the complex, while preventing these putative degradation products and/or non-ADH impurities from interfering with the subunit analysis.
- In future, this methodology should be directly applicable to study a wide range of nocovalent biomolecule interactions.

References

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