Liquid Chromatographic Protein Separation Coupled to Top-down and Bottom-up Mass Spectrometric Analysis

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Overview

- Separation of protein mixtures using intact protein ion exchange and reversed phase separation methodology.
- Analysis of the separated proteins using on and off-line LC-ESI FT-ICR MS.
- Increased depth of analysis of the separated proteins by digestion and 3rd dimension separation of peptides – analysis by ESI-MS/MS.
- Quantitation of samples using SILAC, ICAT and ITRAQ isotopic labelling.

Introduction

Sample complexity is one of the key challenges facing contemporary proteomic analysis. While 2D-electrophoresis is a powerful tool for protein separation, it is difficult to automate, and has limited utility for the analysis of many classes of protein.

Multidimensional liquid chromatography of peptides, exemplified by MuDPIT analysis, is highly automated, but requires the proteolytic digestion of the sample, greatly increasing sample complexity and therefore the required resolution of the subsequent separations. Furthermore, information on differential post-translational modification and isoform expression is often lost.

New developments in column and MS technology have allowed us to perform rapid, improved-resolution, automated separation and MS based identification of intact proteins from complex samples, and very rapid, high resolution peptide separation.

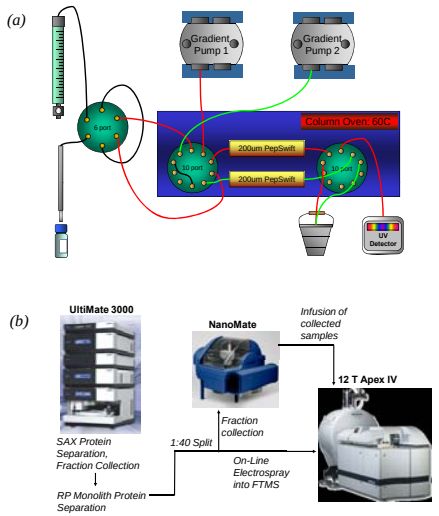
Method

Intact protein separations were performed using a ProPac™ (Dionex) strong anion exchange column for the first dimension, and parallel ProSwift™ (Dionex) reversed phase monoliths as the second dimension.

Second dimension separations were either performed on-line for analysis by FT-ICR MS on a 12T Apex IV (Bruker Daltonics), or off-line into microplates for subsequent digestion and peptide analysis using an HCT Ultra (Bruker Daltonics) for ESI LC-MS/MS.

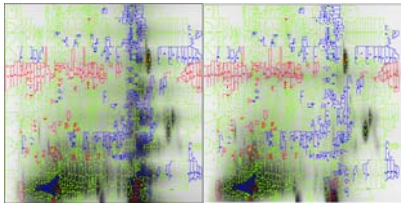
Analysis time peptide ESI LC-MS/MS was decreased by employing parallel 200um PepSwift™ (Dionex) reversed phase monoliths for peptide separation.

Workflow *for 2D-LC intact protein separation analysis by on-line 2D/LC and LC/FT-ICR MS. The first dimension separation is using a 2mm x 25cm anion exchange column coupled to a 2mm x 25cm cation exchange column. The second dimension is performed with a 500um x 5cm PS-DVB monolithic column. For bottom-up analysis, collected fractions are digested with trypsin and analysed either using parallel PS-DVB monolith columns coupled to ion trap MS/MS (a) or by MALDI spotting and rapid ToF/ToF MS. For top-down analysis, fractions are collected during the separation and infused into the FTMS for MS/MS analysis (b).*



Results

Wild-type and pentamidine resistant *Leishmania donovani* were separated using ion exchange and reversed phase intact protein separation methodology.



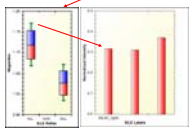
Pseudogels of wild-type (left) and pentamidine resistant (right) L. donovani lysates generated from 2D separation UV data. Y-axis denotes Ion exchange fraction, X-axis is reversed phase time. These maps were generated using the DeCyder DIA software (GE Healthcare).

For **bottom-up** analysis, fractions from the second dimension were collected in microplates, digested (trypsin) and subjected to a 3rd dimension reversed phase LC and analysed by parallel monolith/ESI-MS/MS.

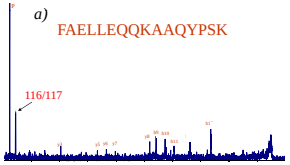
Protein Name and Species	Accession	Score	MW [kDa]	pI	SC [%]	# Pep	# (H/L)	Avg.
nascent polypeptide-associated complex alpha subunit	gi|5031931	322	23	4.36	26	4	1	0.95
nucleobindin	gi|189308	191	53	5.02	9	5	2	1.12
tropomyosin 3 [Homo sapiens]	gi|55665776	145	18	4.56	14	3	1	0.92

Quantitation by isotopic labelling is also applicable to this technique. SILAC labelled lysate from NGF, EGF and untreated transformed human cell line cells was analysed by 2D intact protein separation and parallel LC-MS/MS.

This example shows the ratio of abundance of nucleobindin between the three different cell types.



a)

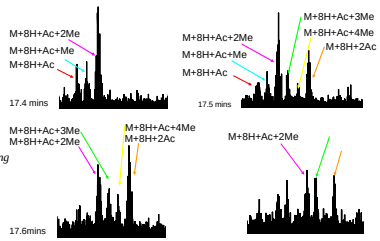


b)

MH+ (calc) [Da]	m/z (calc)	Δ m [Da]	z	RT [min]	Ions Sc.	Rank	S/N	Miss	Sequence	Variable Modifications
1869.86	935.4341	-0.99336	2	4.456339	46.35	1	21.1	0	FLDDDLTDDIMCVK	ICAT-C (C)
1878.89	939.9452	-0.16996	2	4.653391	16	1	36	0	FLDDDLTDDIMCVK	ICAT-C:13C(9) (C)
1885.86	943.4315	0.032322	2	3.773366	58.15	1	1.1	0	FLDDDLTDDIMCVK	ICAT-C (C); Oxidation (M)
2022.98	674.9985	0.883393	3	3.406533	32.15	1	38.9	1	FLDDDLTDDIMCVKK	ICAT-C:13C(9) (C); Oxidation (M)

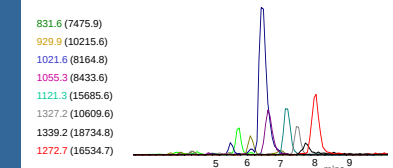
In addition to SILAC labelled samples, iTRAQ (a) and ICAT (b) have been performed on intact protein samples prior to separation using 2DLC of intact proteins. (a) shows an iTRAQ labelled peptide from Leishmania donovani from kinetoplastid membrane protein-11. (b) shows ICAT labelled peptides from alpha-lactalbumin.

For **Top-Down Analysis**, method development was performed using histones, and further developed using lysate from *L. donovani*. After anion exchange separation, fractions were collected and reinjected for further reversed phase protein separation onto a 500um monolith with on-line mass profiling by FT-MS.



Successive FT-MS scans demonstrating chromatographic separation of methylated and acetylated histone H4

ECD fragmentation (for abundant) and CID fragmentation (for weaker precursors) was performed on fractions from *Leishmania*: an example of a fraction containing an 8KDa truncated ubiquitin fusion protein is shown.



Fraction of an intact protein ion-exchange separation,separated by PS-DVB monolith displaying XICs of abundant intact protein charge states.

De-novo sequencing provided a small number of possible targets. ProSight PTM confirmed a match to truncated Ubiquitin Fusion Protein (P-value 1.8x10-96). The protein was truncated from an original mass 14673amu to the observed mass of 8164.4amu. 29 C ions, 30 Z ions matched within 15ppm

C/Z ion matches to the protein

Conclusion

Two dimensional protein separation by liquid chromatography is a high resolution, automated, flexible methodology for proteomic analysis of highly complex samples.

Compatible with a wider range of proteins than 2D gel electrophoresis, this technique allows analysis of hydrophobic, large, basic, and acidic proteins while allowing quantitation by methods such as comparison of UV-map 'pseudo-gels', SILAC, ITRAQ and ICAT.

Additionally, because no digestion is performed prior to separation, this technique allows the possibility of performing further intact protein analyses such as top-down identification.

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