

Mass Spectrometric Quantitation of Differentially Expressed Protein From Stable Isotopically Labeled CHO Cell Media

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Overview

Purpose

- Develop a metabolic labeling protocol to determine the nutritional importance of un-dialyzed vs. dialyzed fetal bovine serum (FBS) by quantitating a recombinantly expressed protein

Methods

- Prepare supplemented Dulbecco's Modified Eagle's Medium containing 10% of either dialyzed FBS with 100 mM fully labeled heavy (¹³C₆, ¹⁵N₂) L-lysine, or un-dialyzed FBS with 100 mM light L-lysine
- Culture Chinese Hamster Ovary (CHO) cells expressing β-galactosidase (β-Gal) containing the FLAG® tag in isotopically labeled and unlabeled media, lyse cells with RIPA Buffer and clarify solution for affinity purification
- Affinity purify samples to capture the FLAG tagged protein using EZview™ Red ANTI-FLAG M2 Affinity Gel
- Elute captured proteins in Laemmli buffer for SDS PAGE
- Perform in gel digest on protein bands using the Trypsin Profile In Gel Digest (IGD) Kit
- Compare differentially expressed protein samples by MALDI-TOF-MS making use of the 8 Da mass shift on lysine peptides both pre and post guanidination

Results

- Stable isotope (SI) labeled media with either dialyzed or non-dialyzed FBS was capable of supporting cell growth
- Cells were lysed efficiently for affinity purification while protease activity was minimized using RIPA buffer containing protease inhibitors
- Affinity purification was successful in capturing the tagged proteins using the ANTI-FLAG M2 Affinity Gel
- Guanidination of lysine C-terminal peptides greatly enhanced ionization efficiency
- MALDI-TOF-MS analysis showed a distinct difference in expression of recombinant proteins when using dialyzed serum; protein ID was successful with 40–50% sequence coverage

Introduction

Recent approaches in analytical biochemistry have made use of stable isotopes for quantitative protein analysis. Several methods of isotope incorporation have been explored including metabolic labeling of cellular proteins using media containing amino acids labeled with stable isotopes.

MALDI-TOF-MS coupled with SI is a powerful technique for relative quantitation and can be used to obtain differential protein expression data or for performing expression profiling. The high purity of modern day SI compounds minimizes isotopic contamination resulting in high quality quantitative data.

The option of using an essential amino acid such as lysine with both nitrogen and carbon SI labels greatly facilitates quantitation. Incorporation of fully labeled lysine results in an 8 Dalton mass shift, which places the isotope population well outside that of the native lysine containing peptide. A deficient CHO cell media was utilized for the selective addition of either fully labeled amino acids or non-labeled amino acids. Mammalian cells previously designed to express β-Gal containing a FLAG epitope tag were used in this study. The experiments described herein are based upon the analysis of recombinant β-Gal with a FLAG fusion tag which was antibody purified using EZview Red ANTI-FLAG M2 Affinity Gel and analyzed by SDS PAGE, and in-gel tryptic digestion. Since the results were unsatisfactory at this point, as only two lysine containing peptides were detected, the signal for lysine C-terminal peptides was enhanced by guanidination¹.

Expression of this protein was used to evaluate different media additives and growth conditions. Mass spectrometric identification and relative quantitation was performed by MALDI-TOF-MS.

Methods

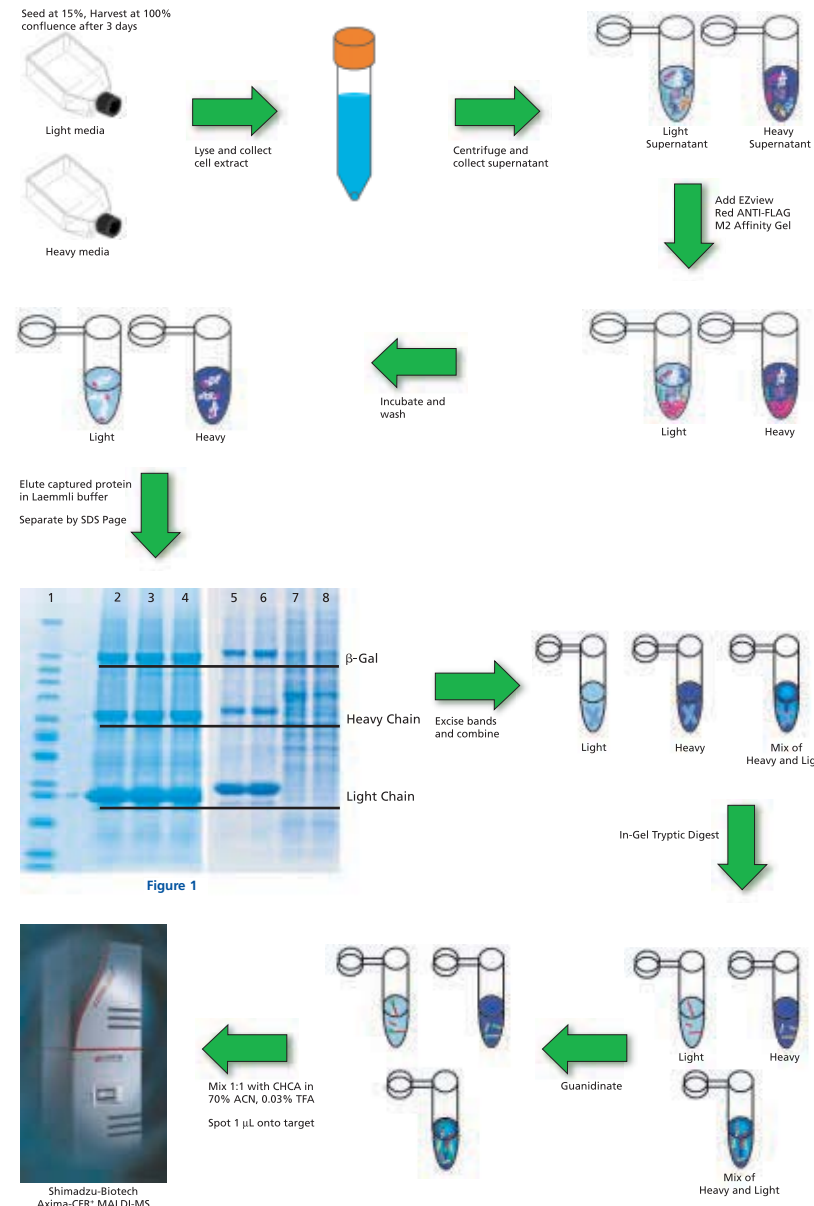


Figure 1



Materials

- Dulbecco's Modified Eagle's Medium/Nutrient Mixture F-12 Ham (D9785, Sigma-Aldrich) all supplements from Sigma-Aldrich
- Fetal Bovine Serum (F2442, Sigma-Aldrich) Dialyzed Fetal Bovine Serum (F0392, Sigma-Aldrich)
- Fully labeled Heavy (¹³C₆, ¹⁵N₂) L-lysine (608041, Isotec); Unlabeled Light L-Lysine (L8662, Sigma-Aldrich)
- Radio-Immunoprecipitation Assay (RIPA) Buffer (R0278, Sigma-Aldrich)
- Protease Inhibitor Cocktail (P8849, Sigma-Aldrich)
- EZview™ Red ANTI-FLAG® M2 Affinity Gel (F2426, Sigma-Aldrich)
- Laemmli Elution Buffer (S3401, Sigma-Aldrich)
- Trypsin Profile In Gel Digestion (IGD) Kit (PP0100, Sigma-Aldrich)
- MALDI matrix α-cyano-4-hydroxycinnamic acid CHCA (C8982, Sigma-Aldrich)
- ProteoMass™ Peptide MALDI-MS Calibration Kit (MS-CAL2, Sigma-Aldrich)
- ProteoMass™ Guanidination Kit (MS0100, Sigma-Aldrich)

Data

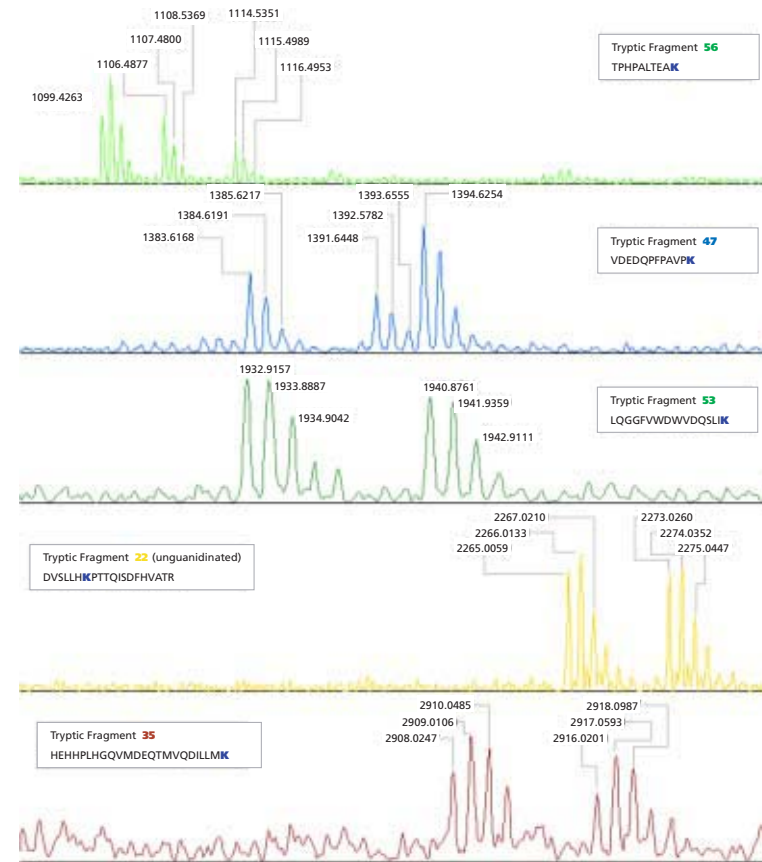


Figure 2: MALDI mass spectra of 5 representative peptide distributions used for relative quantitations (each showing the representative 8 Dalton increase due to SI incorporation). Samples were generated by combining one gel band containing protein composed of endogenous lysine and one band containing fully labeled lysine. These bands were digested and guanidinated then mixed 1:1 with 10 mg/mL, CHCA in 70% ACN, 0.03% TFA prior to MALDI analysis. Spectra were acquired in positive reflectron mode.

Table 1: Un-guanidinated Peak List (40% Sequence Coverage)

Tryptic Peptide	[M+H]	Sequence
9	1428.69	DWENPGVTLQNR
10	1252.66	LAHPFFASWR
16	1067.49	VWGYGQDSR
17	1394.73	LPSEFDLSAFLR
19	801.50	LAVMVLNR
20	1787.73	WSDGSLYEDQDMWR
21	710.37	MSGIFR
22	2265.20	DVSLHKKPTTQISDFHVATR
26	2847.42	VTVSLVQGETQVASGTAPFGGEIIDER
30	1361.72	LWSAEPNLYR
33	1776.11	ENGILLGKPLLR
37	2002.87	CSHPNHPHLYTLCDR
38	2408.14	YGLYVVDANIETHGMVPMNR
40	989.49	WLPAMISR
44	2744.33	NHPSVIVSLGNSGSHGANHDALYR
51	870.43	YVQAFR
54	2446.98	YDENGPNVSAAYGGDFGDTNDR
57	1265.62	HQQQFFQFR
58	1742.90	LSGQTEVSEYLR
60	2517.37	QLELPQPEASAGQLWTLVR
61	2522.23	VVQPNATAWSEAGHSAWQQWR
64	750.37	WQFNR
67	840.53	QLTLRL
69	1457.72	APLNDIGVSEATR
70	1099.55	IDPNNAVIER
73	736.44	TLFSR
76	2466.19	IDGSGQMATVDVEASDTPHPAR
78	1757.86	VNVLGLGPOENYRDR
80	2500.19	WDPLSDMYTPVFPFSENGLR
82	1299.62	ELNYGPHQWR
83	1083.52	GDFQFNISR
84	1507.70	YSQQQLMETSFR
94*	1584.79	RDWENPGVTLQNR
153*	1487.83	QLTLRLDQFTR

Table 2: Guanidinated Peak List (52% Sequence Coverage)

Tryptic Peptide	[M+H]	Sequence
4	1704.27	WVPIVAGLGGGLDTSK
7	826.97	DFVILQNR
9	1429.53	DWENPGVTLQNR
10	1253.45	LAHPFFASWR
12	1101.21	TRPFSQQLR
13	861.93	SLNGEWR
16	1068.13	VWGYGQDSR
17	1395.60	LPSEFDLSAFLR
19	802.07	LAVMVLNR
20	1788.89	WSDGSLYEDQDMWR
21	710.88	MSGIFR
22	2308.90	DVSLHKKPTTQISDFHVATR
23	900.92	FNDDFSR
26	2849.13	VTVSLVQGETQVASGTAPFGGEIIDER
29	856.26	LWVENPK
30	1362.57	LWSAEPNLYR
33	1819.54	ENGILLGKPLLR
35	2910.67	HEHPLHGQVMDQTMVDQLLMK
36	963.04	QNNINAVR
37	2004.26	CSHPNHPHLYTLCDR
38	2409.75	YGLYVVDANIETHGMVPMNR
40	990.17	WLPAMISR
44	2745.97	NHPSVIVSLGNSGSHGANHDALYR
47	1384.82	VDEDQFPFAVPK
51	870.99	YVQAFR
53	1934.50	LQGGFVWDVQSLIK
54	2448.44	YDENGPNVSAAYGGDFGDTNDR
56	1107.55	TPHALTEAK
57	1266.40	HQQQFFQFR
58	1743.95	LSGQTEVSEYLR
60	2518.92	QLELPQPEASAGQLWTLVR
61	2523.77	VVQPNATAWSEAGHSAWQQWR
64	750.84	WQFNR
67	841.04	QLTLRL
69	1458.57	APLNDIGVSEATR
70	1100.22	IDPNNAVIER
73	736.89	TLFSR
76	2467.72	IDGSGQMATVDVEASDTPHPAR
78	1758.93	VNVLGLGPOENYRDR
80	2501.82	WDPLSDMYTPVFPFSENGLR
82	1300.42	ELNYGPHQWR
83	1084.18	GDFQFNISR
84	1508.65	YSQQQLMETSFR
91†	701.15	ELLER
92†	983.16	RDPAVLQNR
94†	1585.72	RDWENPGVTLQNR
129†	3017.25	DRNHPSVIVSLGNSGSHGANHDALYR

Initial peptide mass fingerprinting (PMF) investigation resulted in a sequence coverage of 40% (Table 1) (34 peptides ranging from 700 Da to 3000 Da). This coverage while more than adequate for protein identification, was not sufficient for quantitation in this experiment due to the lack of signal for lysine C-terminal peptides. This difficulty was overcome by converting the lysine residues to homoarginine resulting in enhanced basicity and subsequently better ionization (Table 2). Sequence coverage was enhanced to 52% by guanidination (47 peptides ranging from 700 Da to 3000 Da).

Results

CHO cells were cultured differentially through the addition of either dialyzed or un-dialyzed FBS. The cultures were grown in media containing fully labeled (heavy) lysine or un-labeled (light) lysine. After reaching confluence, the cells were harvested in RIPA buffer containing a protease inhibitor cocktail. The crude cell extracts were affinity purified. Initial confirmation of approximate MW was performed by SDS PAGE. The gel showed elution of the FLAG-tagged protein (Figure 1, lanes 2-6) as well as the heavy and light chain of the M2 antibody (as expected). The FLAG-tagged protein gel bands were excised and combined resulting in a mixture of proteins containing labeled or unlabeled lysine residues. Once the gel bands were in-gel digested, the mixture of peptides was analyzed by MALDI-TOF-MS.

The initial MALDI-MS results produced very poor signal for lysine terminal peptides, making detection and quantitation very difficult.¹ In order to overcome this difficulty, the lysine C-terminal peptides were guanidinated resulting in superior sequence coverage through detection of seven additional lysine terminal peptides (Table 1 and Table 2). Detection of these peptides also provided more convincing quantitation of the differentially expressed protein (β-Gal).

The media employed in this study was supplemented with either dialyzed or non-dialyzed fetal bovine serum (FBS) in order to assess the effect on cell growth and protein expression. The dialysis performed (ultra filtration) effectively removed all lysine. In order to confirm the absence of any detectable lysine, serum samples were analyzed using the Waters ACQ-Tag system. Amino acid content was calculated at 25 mg/L in the un-dialyzed FBS and was not detectable in the dialyzed FBS.

While the "contamination" with native lysine is negligible (2.4%), the other nutrients in the FBS do seem to have an effect on protein expression. Quantitation was performed by summing peak areas for the lysine containing peptides and comparing those summed areas to those in the SI incorporated distribution (seen 8 Da higher Figure 2). The effect of dialyzed FBS was to reduce protein expression (Figure 3), over 8-fold more than can be accounted for by the endogenous free lysine (2.4%).

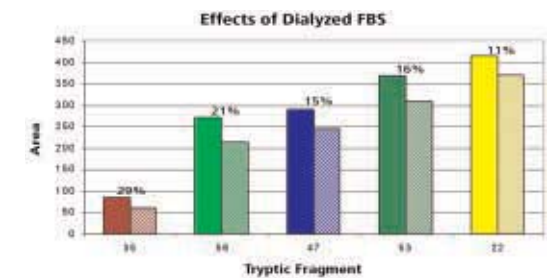


Figure 3: Monoisotopic peak area of five lysine containing tryptic fragments with (shaded) and without SI incorporation. Listed above each set of bars is the percentage decrease in peak area when using the dialyzed FBS.

Conclusions

- The use of dialyzed FBS did not adversely affect cell growth but did reduce protein expression by approximately 19%
- Fully labeled lysine is appropriate for use in a quantitative study of protein differential expression
- Affinity purification of FLAG-tagged proteins was simple and efficient using the EZview Red ANTI-FLAG M2 Affinity Gel
- Guanidination of protein tryptic digests enhanced sequence coverage and facilitated quantitation
- MALDI-TOF MS is a powerful tool for comparative protein expression studies when coupled with SI metabolic labeling

References

- Jungblut, P, et al., The Dominance of Arginine-Containing Peptides in MALDI-Derived Tryptic Mass Fingerprints of Proteins. Anal. Chem., **71**, 4160-4165 (1999).