Characterization of Heavy Recombinant Proteins for Use as Internal Standards in Quantitative MS Workflows

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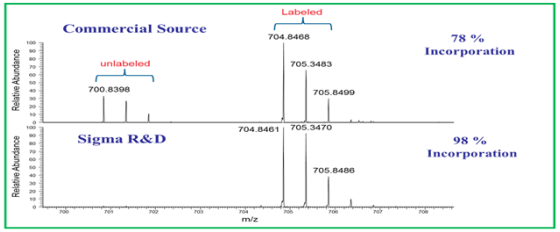
Overview

Objective: Characterization of heavy (SIL) proteins for use in quantitative MS.

Introduction

Stable isotope labeled (SIL) or heavy standards have been used in quantitative MS for several decades. In the proteomics area, heavy labeled peptides are the primary source of heavy labeled standards. The main disadvantage of the labeled peptides arise from the fact they are spiked into the sample late in the sample preparation workflow and therefore do not account for variability in steps such as digestion or protein enrichment/fractionation. The ideal internal standard would be an stable isotope-labeled full length protein that is equivalent to the native target protein. The SIL protein can then be introduced at the beginning of the workflow and should account for any variability during sample preparation. We have demonstrated the capability to produce SIL proteins of >95% heavy isotope incorporation and purity >95% by SDS-PAGE. We have also investigated glycosylation and binding properties to polyclonal antibodies, as well as studied the digestion time course to determine if the heavy protein has the same digestion profile over time as the endogenous protein.

SIL Incorporation



Peptide Sequence	Sigma R&D	Commercial Source
DYVSQFEGSALGK	97.8%	77.7%
THLAPYSDELR	98.0%	71.2%
VQPYLDDFQK	96.0%	75.2%
Average	97.3%	74.7%

Protein	% SIL	Protein	% SIL
AKT1	>98%	EPO	>98%
MP2K4	>98%	APOD	96.8%
HPX	>98%	TGFA	96.0%
STAM1	97.8%	TGFB3	94.4%
IL6RA	>98%	APOA1	97.3%

Figure 1. Incorporation of stable isotopes for several peptides of SIL-APOA1 from a Sigma R&D batch and a commercially sourced material. Analysis was performed on a LTQ-FT (Thermo). No conversion from heavy Arg to heavy Pro was observed. Several other proteins were also analyzed with stable isotope incorporations of >94%.

Intact Mass Analysis of SIL-APOA1

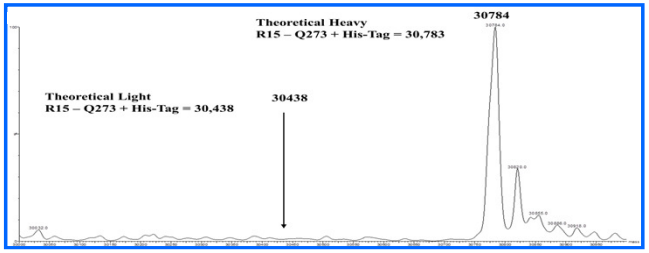


Figure 2. The intact mass of SIL-APOA1 was measured by SEC-MS using a 2 mm X 30 cm SEC TOSOH Bioscience (# 21485) column using 45% ACN 0.1% FA buffer. The MS data was collected on a Q-TOF Premier (Waters) and deconvulted using MaxEnt1. The intact mass was found to be 30784Da, 32 ppm different from the theoretical value. No lock mass was used for the analysis.

SIL-APOA1 Sequence Coverage

RHFWQQDEPPQSPWDRVKDLATVYVDVLKDSGRDYVSQFEGSALGKQLNL
KLLDNWDSVTSTFSKLREQLGPVTQEFWDNLEKETEGLRQEMSKDLEEVKA
KVQPYLDDFQKKWQEEMELYRQKVEPLRAELQEGARQKLHELQEKLSPLGE
EMRDRARAHVDALRTHLAPYSDELRQRLAARLEALKENGGARLAEYHAKAT
EHLSTLSEKAKPALEDLRQGLLPVLESFKVSFLSALEEYTKKLNTQ

Figure 3. Sequence coverage (red) of SIL-APOA1 digested with trypsin and analyzed by LC/MS^e on a Q-TOF Premier (Waters). All spectra were manually verified.

Binding Kinetics of SIL-APOA1 vs Native APOA1

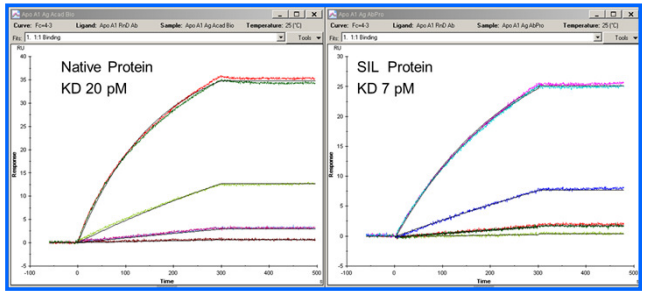


Figure 4. Binding curves for SIL-APOA1 and APOA1 isolated from sera (Academy Bio Medical #11P-101) with a goat polyclonal AB (R&D Systems AF3664). Data from a Biacore T100 (GE). There is very little difference between the binding of the native protein and the SIL protein. The difference in the KD measurements are due to the time for dissociation not being long enough to have an accurate measurement of K_d.

SIL-APOD Glycosylation Observed

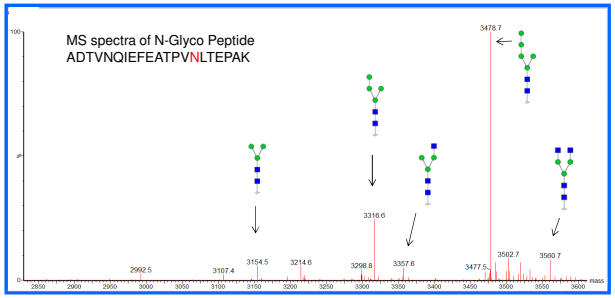


Figure 5 . MS and MS^e of the N-glyco peptide ADTVNQIEGEATPVNLTEPAK from SIL-APOD deconvoluted using MaxEnt3. A series of ions were observed consistent with a typical N-glyco peptide. The peptide sequence was verified by MS^e.

SIL-APOA1 Digestion in Human Sera

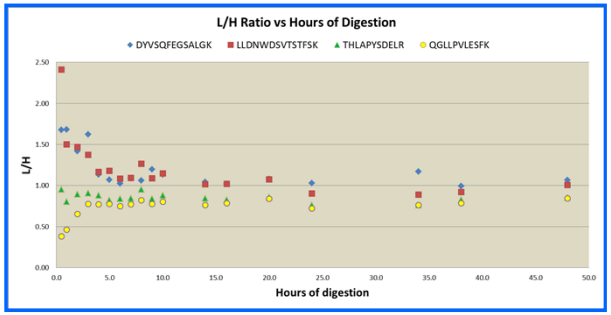


Figure 6. SIL-APOA1 protein was spiked into human serum at approximately a 1:1 ratio with the endogenous protein. The serum was digested with trypsin with aliquots pulled from 0.5 hours to 48 hours. Peptides were monitored by MRM, (5500 Q-Trap AB-Sciex). The L/H ratio is plotted as a function of digestion time. After ~8 hours the L/H ratio becomes constant. At early digestion times the ratio deviates from the expected value for some of the peptides. This initial deviation may be due to differences in denaturation of the heavy and endogenous proteins. Use of a harsher denaturation buffer may eliminate the deviation observed at early digestion times.

Summary

We have characterized several heavy proteins looking at purity, incorporation of the heavy isotope, PTM's, binding profiles to antibody and digestion time course profiles. The heavy proteins were shown to be sufficiently similar to be useful as internal standards for quantitative MS.

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