POSTER NOTE

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Development of an All-Recombinant Intact Protein Standard for LC-MS Application Development and System Suitability Testing

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OVERVIEW

Purpose : develop a high quality, reproducible intact protein standard for LC-MS QC and application development.

Methods : expression and purification of recombinant proteins that meet MW, m/z distributi purity, stability, reproducibility requirements of a sample used for LC and MS QC.

Results: a high quality, recombinant intact protein mixture for LC-MS quality control and application development was developed.

INTRODUCTION

In recent years, interest in intact protein analysis by HPLC, LC-MS, and MSMS has increas significantly. This can be attributed to both improvements to LC and MS hardware, instrume control software, and data processing software. Conceptual shifts in how we can best addr and answer biological questions given these emerging commercially available capabilities a explain this paradigm shift. Having witnessed the explosive growth of bottom-up proteomics and the subsequent evolution of high-quality, widely accessible standards to normalize platform performance in time and space, and assist with method development for new applications, we recognize a similar need for the Top-down proteomics field. Here, we describe the development of a high quality, recombinant, multi-purpose intact protein stand for LC, LC-MS, and LC-MSMS quality control and application development.

MATERIALS AND METHODS

Protein standards were purchased from Sigma-Aldrich and evaluated by direct infusion ESI-MS on a Thermo Scientific™ Q Exactive™ hybrid quadrupole-Orbitrap™ MS. Seven proteins were selected that 1) evenly covered a MW range of 12kD – 66kD, 2) presented mostly clean, modification and adduct-free ESI spectra, and 3) whose ESI charge state distributions covered a wide m/z range from 500-2000. Mixing ratios were adjusted such that all seven proteins could be detected simultaneously in a single infusion MS experiment (R&D standard).
A number of proteins candidates that were expected to mimic the characteristics of the proteins in the original R&D standard were then expressed in *E. coli and B. subtills* and purified. All were screened for ionization efficiency, purity, MW, charge state distribution by LC-MS, and infusion-MS, resulting in selection of the final candidates for the recombinant standard. Quality and stability of the selected proteins were verified by SDS-PAGE, UV HPLC, infusion-MS and LC-MS, and MSMS using a Q Exactive mass spectrometer. High resolution HCD spectra were collected to confirm the sequences assigned to the final protein list. From these confirmed sequences a standard FASTA file and flat file were created for distribution and easy analysis.
Mixing ratios were again optimized and stability of the mixture was confirmed, again by SDS-PAGE, UV HPLC, infusion MS, and LC-MS.

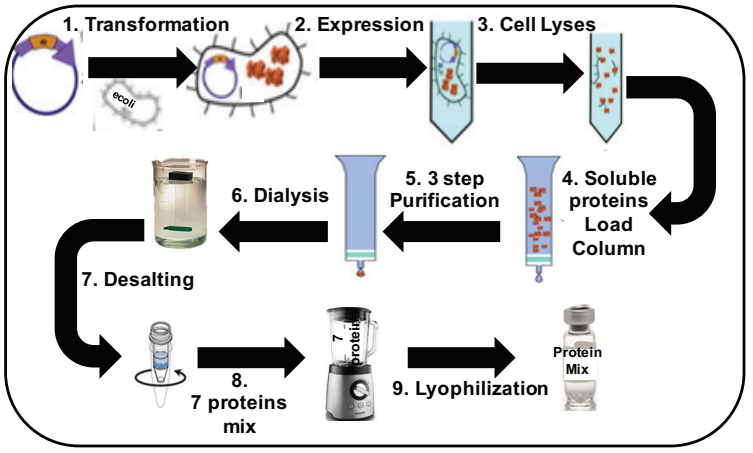

Figure 1. Workflow for recombinant protein mixture generation.

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RESULTS – Preliminary R&D Mixture

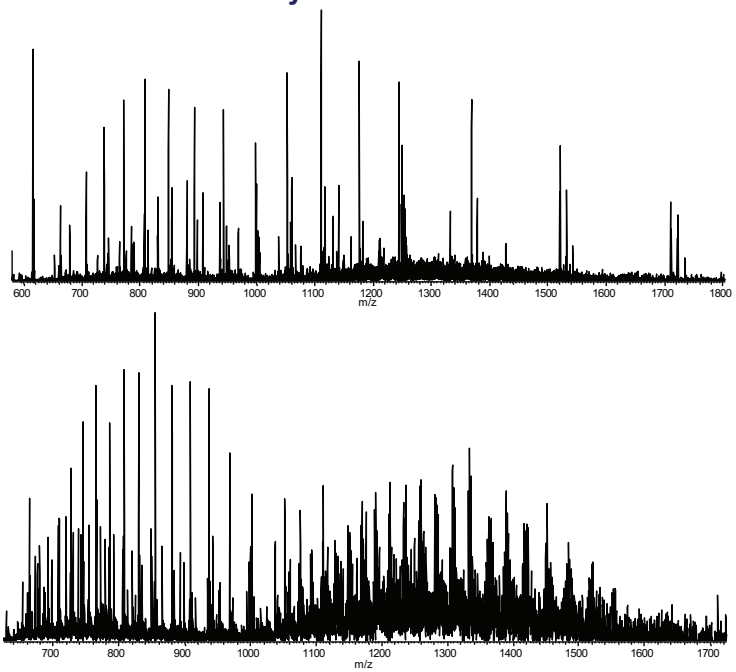


Figure 2. Original R&D mixture infusion-MS spectra. Top panel – infusion spectra of R&D mixture at 140k resolution. Proteins from 12kD to 29kD are routinely detected. Bottom panel – infusion spectral of R&D mixture at 8k resolution. All proteins typically detected, including the proteins at 42 and 66kD.

Table 1. Original R&D mixture composition, MW range, and m/z range

Protein	Sigma PN	~MW (kD)	Center m/z
Cyochrome c	C2506	12	773
RNAse A	R6513	14	1369
Myoglobin	M0630	17	808
Trypsin Inhibitor	T6522	20	1110
Carbonic Anhydrase	C2522	29	830
Enolase	E6126	42	771
BSA	A5611	66	1210

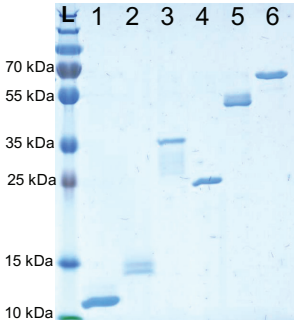
The original protein mixture met the sample requirements of wide molecular weight range, widely distributed charge state envelopes across m/z, and representative diversity for quality control, application development, and method optimization. The R&D mixture, however, did not meet regulatory requirements with respect to animal origin components in the mixture, and no stability studies were done. In fact, degradation of certain proteins during storage was observed over time.

RESULTS – New Recombinant Mixture

Figure 3. SDS-PAGE of several recombinant candidates for MS analysis.

1 – Thioredoxin (Trx)
2 - RNAseA
3 - Protein G
4 - Carboic Anhydrase
5 - Protein AG
6 – Klennow

350 ng samples loaded to 12% SDS gel



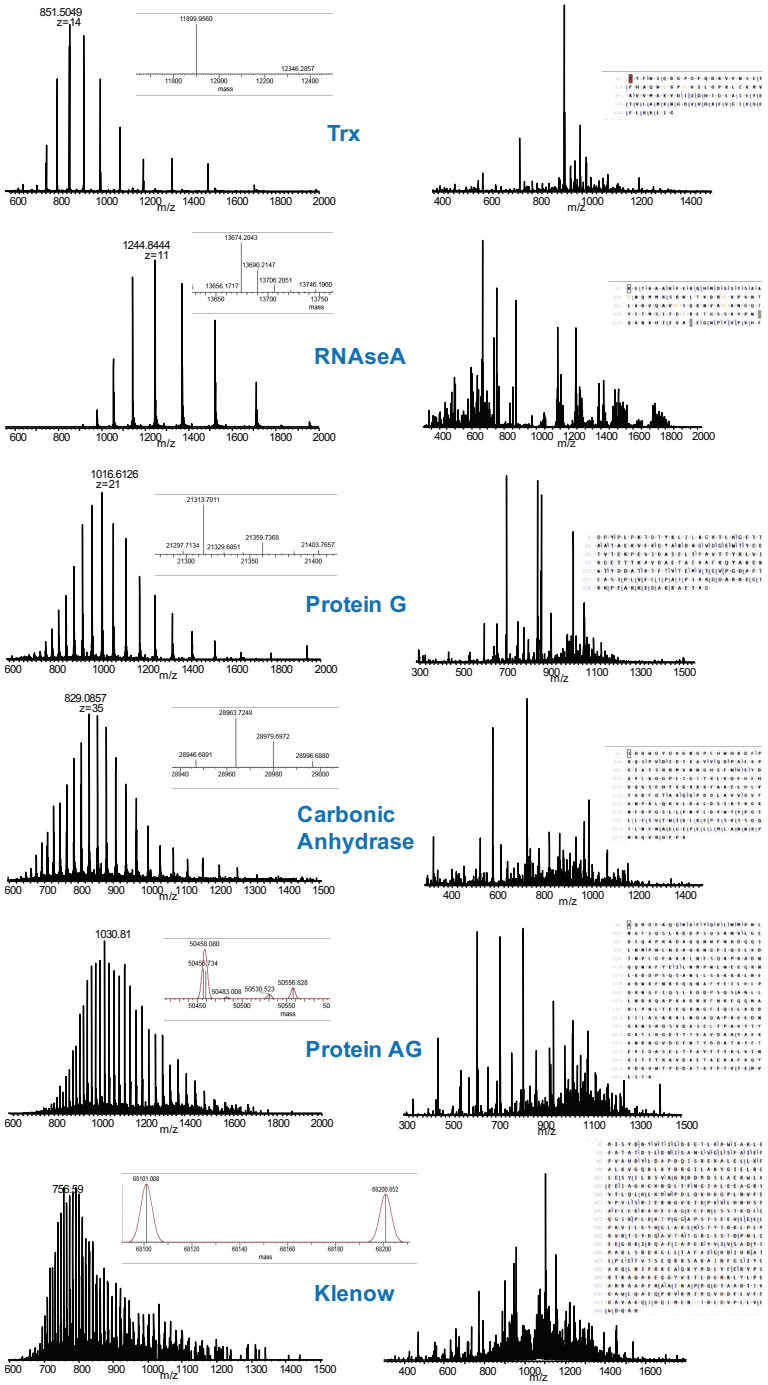


Figure 3. MS analysis of protein candidates. Left panel shows the full scan spectrum of each candidate protein collected on a Thermo Scientific™ Q Exactive™ HF MS with an inset showing deconvolution results from Thermo Scientific™ Protein Deconvolution 3.0 software. In some cases, oxidation or sodiation are evident. Work is ongoing to reduce sodiation. The right hand panel shows HCD spectra of each protein collected on a Q Exactive HF along with Thermo Scientific™ ProSightPC 3.0 software results (inset). The ProSight results from the largest protein, Klennow, were derived from a high resolution CID spectra from the Thermo Scientific™ Orbitrap Fusion™ Lumos™ Tribrid™ MS.

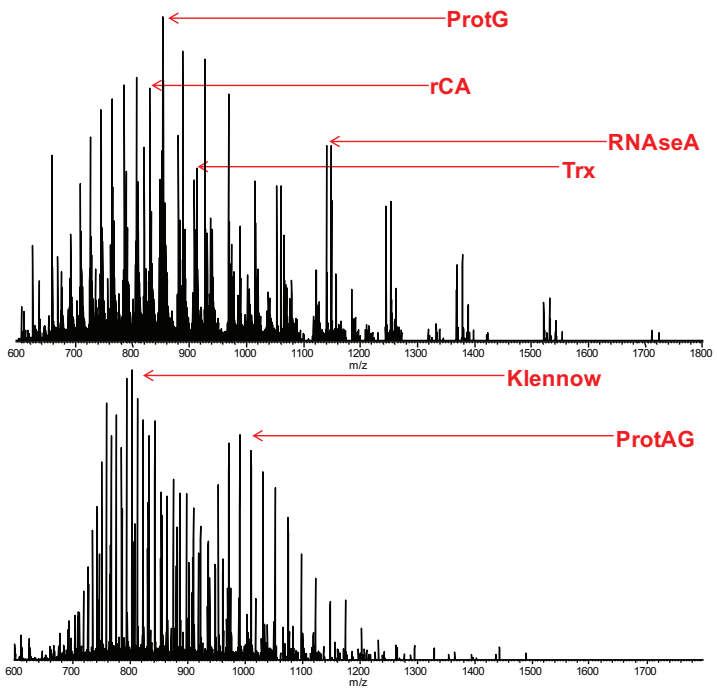
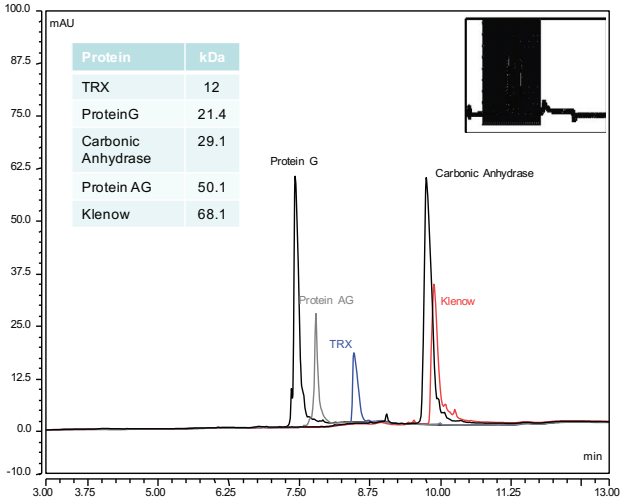
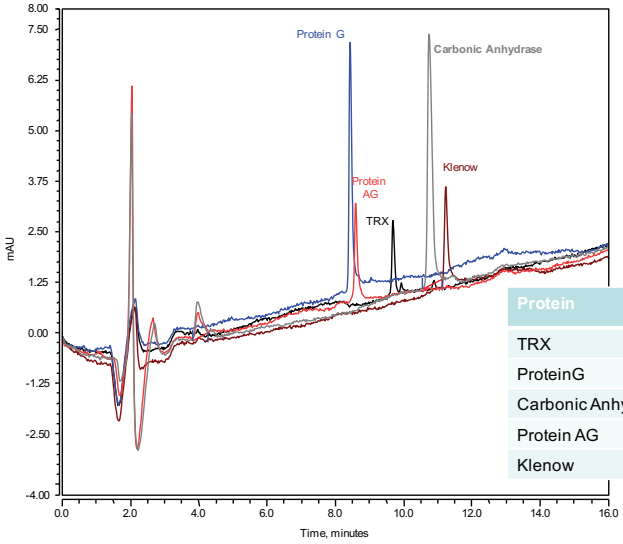


Figure 4. Final recombinant mixture infusion-MS spectra. Top panel – infusion spectra of the optimized mixture using new recombinant protein candidates at 140k resolution. Trx, RNAseA, Protein G, and Carbonic Anhydrase are detected at this resolution setting. Bottom panel – infusion spectral of new candidate mixture at 8k resolution. All proteins typically detected, including larger proteins, Protein AG and Klenow.



Protein	kDa
TRX	12
ProteinG	21.4
Carbonic Anhydrase	29.1
Protein AG	50.1
Klenow	68.1

Figure 5. UV-HPLC analysis of recombinant proteins. MAbPac RP 2.1 x 50mm column; mobile phase A: Water + 0.1% Formic acid, B: 80/20 (v/v) ACN/ Water + 0.1% Formic acid; Flc Rate: 0.3 mL/min; 5-80% B in 15 minutes; Temperature: 80C; Sample: 0.2mg/mL protein (see chromatogram); Injection: 10 µL; Detection: UV 280 nm.



Protein	kDa
TRX	12
ProteinG	21.4
Carbonic Anhydrase	29.1
Protein AG	50.1
Klenow	68.1

Figure 6. UV-HPLC analysis of recombinant proteins. Thermo Scientific™ UltiMate™ 3000 RSLCnano System; Column: Thermo Scientific™ ProSwift™ RP-4H LC 200µm x 25cm; Mobile Phase A: Water + 0.1% Formic acid, B: 80/20 (v/v) ACN/ Water + 0.1% Formic acid; Flow Rate: 5 µL/min; 5-80% B in 15 minutes; Temperature: 30° C; Sample: 0.2mg/mL protein (see chromatogram); Injection: 0.1 µL. Detection: UV 214 nm

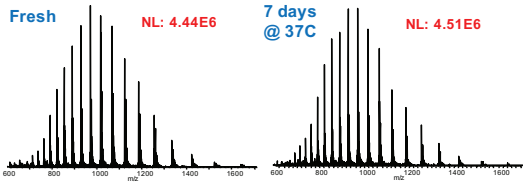


Figure 7. Preliminary stability studies. Shown here is Protein G, reconsituted fresh or after 7 days incubated at 37C, corresponding to 1 year storage at -20C.

CONCLUSIONS -

A high quality intact protein standard was developed that meets needs for HPLC, MS, LC-MSMS quality control, method development, and optimization. The completely recombinant nature of the sample makes it compatible with clinical applications. The proteins cover a wide MW range, m/z range, and demonstrate good chromatographic separation.

ACKNOWLEDGEMENTS -

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