71st ICoMST
GIRONA - CATALONIA - SPAIN
3rd-8th August - 2025

PEPTIDE QUANTIFICATION IS ESSENTIAL FOR BIOLOGICAL MARKER CHARACTERIZATION IN POST-RIGOR MUSCLE

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I. INTRODUCTION

Mass spectrometry-based proteomics has allowed the characterization and discovery of protein markers for numerous traits, conditions, and diseases. A biological marker must be specific to routinely and consistently be a target for evaluation. While proteomics broadly characterizes 'proteins', the actual unit of measure in mass spectrometry-based systems stems frequently from peptide-spectral matches, ie, tryptic peptides. Biological differences can be masked when summarizing at the protein group level, especially in protein groups with more identified peptides, as more diverse sets of peptides may not always change in similar directions [1]. Recognizing and appreciating that these measurements must ultimately be evaluated on the peptide level is essential, especially in postmortem muscle. Unregulated postmortem cellular environments impact protein solubility and integrity by altering pH and the activity of proteinases. Specific proteolytic events target key muscle proteins, producing varying polypeptide units that are smaller in molecular weight and likely have altered isoelectric points and hydrophilic attributes compared to the intact parent protein. Interpreting proteomic results from postmortem and post-rigor muscle at a protein-level often overlooks important details about the differences and changes of specific tryptic peptides. These details are important for guiding future studies and developing better prediction models. Therefore, the objective was to quantify and compare differences at the peptide-level of two soluble protein fractions from pork loins categorized by postmortem pH. It was hypothesized that this analysis would reveal specific tryptic peptides that overlap in differences between protein fractions and time points that can be used as specific biological markers.

II. MATERIALS AND METHODS

Pork loins were evaluated and selected based on pH at 1 d postmortem in a commercial plant [2]. Two balanced categories (n = 25/category) were generated to establish samples with differences in meat quality: Low pH (5.59–5.71) and High pH (5.90–6.46). Individual chops were aged for 1, 7, or 14 d postmortem, and frozen (-80°C) after their respective aging periods. Two separate proteomic experiments were conducted, where each pork loin sample and aging period was analyzed using two different starting protein fractions: 1) proteins extracted from muscle tissue in a low-ionic strength buffer (50 mM Tris-HCl [pH 8.5], 1 mM EDTA) and 2) proteins collected from muscle exudate. Briefly, proteins soluble in a low-ionic-strength buffer were extracted from powdered muscle tissue samples [3]. Muscle exudate samples were clarified by centrifugation and diluted 1:7 (vol/vol) in a low-ionic-strength buffer. All samples within protein fractions were diluted to equivalent protein concentrations before proteomic analysis. Proteins were reduced, alkylated, and digested with trypsin. Within each protein fraction, tryptic peptides were labeled with Tandem Mass Tag (TMT) 10plex reagents (Thermo Scientific), and a pool of all samples within protein fraction was labeled with a TMT11-131C (Thermo Scientific) reagent to serve as an internal reference. Peptides were separated by liquid chromatography over 4.5 h and introduced into a Q-Exactive Hybrid Quadrapole-Orbitrap mass spectrometer (Thermo Scientific) [3]. The resulting .raw files were converted to .mzML using msConvert and analyzed with FragPipe (v. 22.0) using default settings against a *Sus scrofa* UniProt reference proteome .fasta file (22,673 sequences). Peptide spectrum match files were summarized and analyzed using MSstatsTMT (v. 2.14.2) with default settings. Tryptic peptides in < 60% of samples within a time point were excluded. Fitted mixed models were used to evaluate pairwise comparisons of pH category within protein fraction and time point. Peptides were considered significantly different at a Benjamini-Hochberg false discovery rate (FDR) < 0.05 and $\log_2$ fold difference > |0.3|. Pearson correlations (r) of peptide abundance between protein fractions within a time point were calculated.

III. RESULTS AND DISCUSSION

The High pH category was characterized by a trained panel as more ($P < 0.05$) tender, juicy, less ($P < 0.05$) chewy, and had lower ($P < 0.01$) instrumental star probe tenderness at each day postmortem [1]. Variation in water holding capacity was observed, where the High pH category had less ($P < 0.01$) cook loss and drip loss than the Low pH category [2]. 834 tryptic peptides were detected at all time points in both protein fractions. Figure 1 highlights that 48 tryptic peptides were differentially abundant (FDR < 0.05) between the High and Low pH categories across all time

points between the two protein fractions. Unsurprisingly, the protein-level differences from our companion short paper align with the peptide-level differences. This current approach highlights the specific peptide targets that can be utilized in subsequent targeted experiments. The correlation of tryptic peptides between protein fractions within a time point is strong (r = 0.64–0.69, Figure 2), illustrating that their abundance between protein fractions is similar. More revealing is that several tryptic peptides are revealed to be differentially abundant (FDR < 0.05) between the High and Low pH categories across all time points and between protein fractions. These tryptic peptides arise from calcium-transporting ATPase, filamin C, fructose-1,6-bisphosphatase isozyme 2, titin, and tripartite motif-containing protein 72. On a protein-level, the magnitude of difference in titin abundance regardless of protein fraction and time point is low (log_2 fold difference ~ 0.25). This current analysis on a peptide-level revealed tryptic peptides that were consistently greater in abundance (log_2 fold difference ~ 1.0) in the High versus Low pH category across all time points and protein fractions. This demonstrates that summarizing proteomic data to a protein-level can mask the magnitude of difference in some proteins in ways that overlook valuable results and potential biological markers.

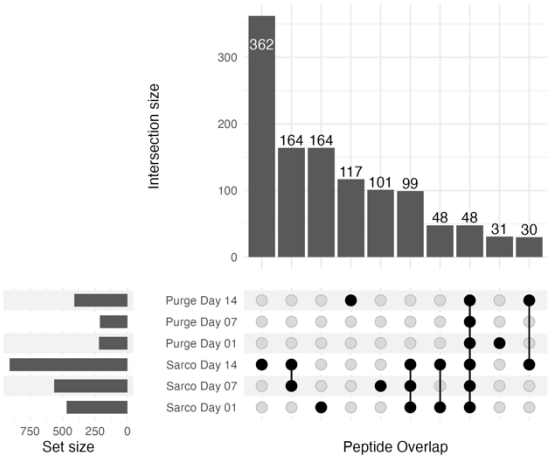


Figure 1. Comparison of significantly different tryptic peptides between protein fractions over postmortem time points.

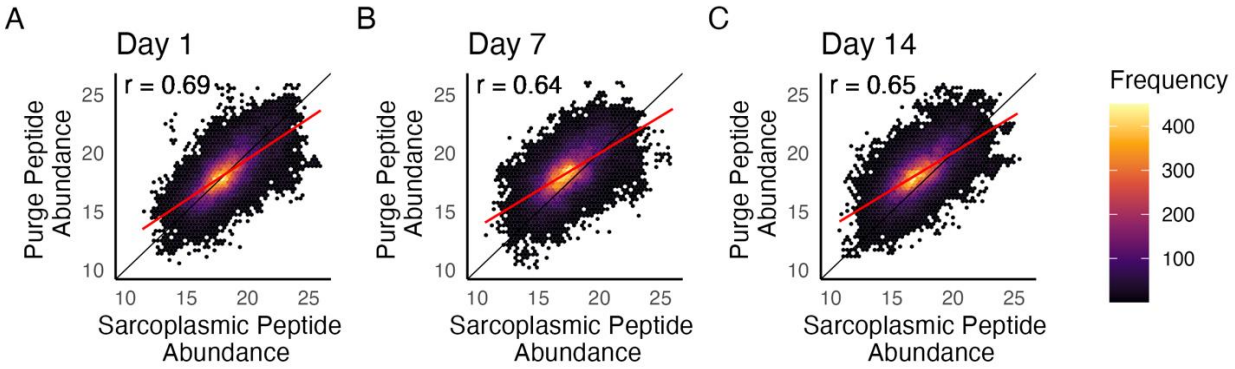


Figure 2. Pearson correlations (r) of tryptic peptides between the sarcoplasmic muscle extracts and the muscle exudate at Day 1 (A), Day 7 (B), and Day 14 (C). The black solid line is y = x, and the red solid line represents the linear relationship between sarcoplasmic and purge. Peptide abundance is on the log_2 scale.

IV. CONCLUSION

This work sought to define tryptic peptide markers of key proteins that can be used to evaluate the ultimate meat quality. Distinct from most proteomic summarizations, this work quantified differences in tryptic peptides, which revealed relevant differences in proteins, like titin, that are more muted when summarized on a protein-level. These data illustrate that in postmortem muscle, proteomic studies, a deeper understanding and more robust prediction models could be gained by consideration of changes in specific tryptic peptides.

ACKNOWLEDGEMENTS

This work was supported, in part, by the United States Department of Agriculture–Agriculture and Food Research Initiative project 2019-67017- 29181, the National Science Foundation under Grant No. DGE-1828942, and the Iowa Pork Producers Association.

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