PROTEOMIC ANALYSIS OF THE YEAST RIBOSOME

By

Daniel T. Ladror

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The dissertation is approved by the following members of the Final Oral Committee:

Lloyd M. Smith, Professor, Analytical Chemistry

John C. Wright, Professor, Analytical Chemistry

David C. Schwartz, Professor, Analytical Chemistry

Clark R. Landis, Professor, Organic Chemistry

Etienne Garand, Assistant Professor, Physical Chemsitry

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PREFACE

The work presented in this dissertation was performed at the University of Wisconsin-Madison under the supervision of Professor Lloyd M. Smith. It describes the proteomic study of yeast ribosomes as a biological molecular machine of interest, as well as a useful model for testing mass spectrometry techniques.

Chapter 1 gives a brief introduction on proteomics, mass spectrometry, and ribosomal structure and function. Chapter 2 discusses using bottom-up mass spectrometry to examine the effect of growth conditions on yeast ribosomal protein methylation and acetylation. Chapter 3 describes using chemical charge tags to enhance ETD fragmentation of yeast ribosome peptides in order to improve sequence coverage. Chapter 4 discusses unpublished experiments to optimize pre-fractionation and top-down mass spectrometric analysis of ribosomal proteins. Finally, chapter 5 discusses contributions made to a fluorescence detection system that was published in Analytical Chemistry, volume 83, pages 2187-2193, 2011 under the title "Parallel Detection of Intrinsic Fluorescence from Peptides and Proteins for Quantification During Mass Spectrometric Analysis."[1]

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I also wish to thank to my advisor, Lloyd Smith, for the guidance, funding, and opportunity to pursue research in his lab for five years. Lloyd has an excellent knack for viewing the big picture and teaching others to do so as well. It is thanks to him that I can say I "feel like a scientist."

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Daniel T. Ladror

Under the supervision of Professor Lloyd M. Smith

University of Wisconsin-Madison

ABSTRACT

Ribosomes are the center of protein translation in the cell, but the biological roles of ribosomal post-translational modifications (PTMs) are largely not understood. Synthesis of new ribosomes consumes the majority of a cell's energy during exponential growth. Thus, careful regulation of ribosome synthesis and function is essential for cell survival. Ribosomal regulation has been found to be influenced by a number of environmental conditions, including nutrient availability, environmental stressors and growth phase. In this work, we examine regulation of ribosomes by using mass spectrometry to quantify the post-translational modifications on yeast ribosomal proteins under different growth phase conditions.

Mass spectrometry is a central tool of proteomics. The standard proteomics experiment involves tryptic digestion of the proteins to be analyzed, followed by mass analysis and identification of the peptides, in what is called bottom-up mass spectrometry. The bottom-up approach benefits from robustness and ease of use, but not all peptides are amenable to ionization or detection, which limits sequence coverage and the number of identified proteoforms (e.g., isoforms from alternative splicing, protein variants arising from genetic variation, and/or proteins with various post-translational modifications). Thus, several different

methods are commonly employed by the proteomics community to improve sequence coverage including pre-fractionation, optimized chromatographic separation, and chemical derivatization of peptides and proteins. We have utilized chemical derivatization to improve Electron Transfer Dissociation (ETD) fragmentation for ribosomal peptides, with the goal of increasing sequence coverage and consequently, the number of PTMs identified.

Even with improved sequence coverage, bottom-up mass spectrometry sacrifices contextual information about the entire protein. For example, proteoforms cannot readily be identified at the peptide level, especially if a specific codon substitution or PTM is located on a tryptic peptide that is not easily detected. Thus, in order to obtain quantitative information of ribosomal proteoforms, the ribosomal proteins must be analyzed intact. In this study, we discuss optimization of methods to isolate, fractionate, and analyze intact ribosomal proteins. We also explain steps taken to optimize a system designed to allow quantification by intrinsic fluorescence in tandem with identification by mass spectrometry, with the goal of achieving more accurate and precise protein quantification.

CHAPTER 1:

INTRODUCTION

The foundation of biology entails that genetic information stored in an organism's DNA is used to synthesize RNA and protein which conduct the functions of a cell. However, the proteins in an organism cannot be predicted entirely from the DNA, due to alternative splicing, post-translational modifications, and single nucleotide polymorphisms.[2-4] Thus, to fully understand an organism on the protein level, the proteins must be analyzed directly.[5]

This development has given rise to the field of proteomics, fueled in part by advancements in mass spectrometry. Proteins and peptides can now be ionized by soft ionization methods such as electrospray ionization (ESI), where a voltage is applied to a narrow tip from which analyte solutions are eluted, and matrix-assisted laser desorption ionization (MALDI), where analytes are mixed with a matrix, deposited on a metal plate, and moved to the gas phase by ionizing the matrix with a laser. These methods ionize most peptides and proteins intact, allowing them to be analyzed in the gas phase by a variety of mass spectrometers, including triple quadrupole (QQQ), time-of-flight (TOF), ion trap, orbitrap, and fourier transform ion cyclotron resonance (FTICR). Sequencing data can be acquired by fragmenting peptides and proteins using collision induced dissociation (CID) or electron capture/transfer dissociation (ECD/ETD) and analyzing the masses of the resulting fragments. This is especially useful for identifying and localizing post-translational modifications (PTMs) such as methylation, acetylation, and phosphorylation. TOF, orbitrap and FTICR mass spectrometers are the best suited for analyzing larger molecules like proteins, but orbitrap and FTICR instruments are best equipped to fragment those proteins and obtain MS/MS data. Currently, orbitraps are generally

suited to analyze proteins up to 30 kDa, while FTICR instruments are generally suited to analyze proteins up to 50 kDa, although far larger proteins have been successfully analyzed using specialized methods.[6, 7] However, orbitraps are significantly cheaper, and thus more accessible to a wider range of laboratories, than FTICR instruments.

One of the most common proteomics procedures is a "shotgun proteomics" experiment, which involves enzymatically digesting the proteins of a biological sample, separating them with a reverse-phase capillary HPLC column, and ionizing them by ESI to be analyzed by a mass spectrometer.[8, 9] This setup is useful for its ability to analyze a large number of peptides in a single sample with minimal manual preparation, but it does have its limitations. When analyzing sufficiently complex samples, different analytes may coelute. This may lead to signal suppression, where the more readily-ionizing analyte consumes the available charge and is detected with strong signal, while the other analyte is detected with little to no signal.[10] Furthermore, even if multiple coeluting analytes all ionize sufficiently, the mass spectrometer is limited in how many peptides it can isolate for fragmentation, forcing it to isolate some candidates while leaving others unidentified. To avoid this, one may pre-fractionate complex samples into several fractions of reduced complexity; however, unavoidable sample loss causes problems for quantitative measurements. Most molecules experience imperfect ionization efficiency, meaning not all of the instances of a molecule that are ejected from the electrospray ionization tip successfully become gas-phase ions.[11] Furthermore, platform-specific mass spectrometer biases may be introduced, making cross-platform quantification comparisons difficult.[12] Because of this, most quantitative measurements by mass spectrometry are relative measurements, where the quantity of an analyte detected is compared to that of a different sample, or to a standardized curve. Biological samples can be labeled with heavy and light

isotopes in a variety of ways, but label free quantification has also been found to be reliable for quantifying changes in abundance of two-fold or greater.[13]

One way to avoid the problems that arise for a complex sample is to focus on a subset of the proteome. This is commonly done by targeting the proteins of a specific organelle or region of the cell, such as the ribosome. A full yeast digest contains thousands of proteins, while the yeast ribosome only contains about 79 proteins.[14] This makes the ribosome a suitable candidate for proteomic analysis.

As the center of protein translation in the cell, the ribosome is an interesting topic in its own right. Ribosomes are ribonucleoprotein complexes that are inherently conserved among prokaryotic and eukaryotic species. Pre-rRNA molecules are synthesized in the nucleolus and transported to the nucleus, while the ribosomal proteins (RPs) are translated by existing ribosomes in the cytoplasm and transported to the nucleus. Within the nucleus, RPs bind the 5S, 5.8S, 18S, and 28S rRNA to form pre-subunit complexes. These complexes are then exported from the nucleus to be assembled into the 40S small subunit and 60s large subunit in the cytoplasm just outside of the nucleus.[15, 16]

The enzymatic function is performed by the rRNA molecules, with the ribosome proteins (RPs) generally thought to provide structural support for the rRNA. It is for this reason that the overwhelming majority of ribosome studies focus on the rRNA. However, RPs have been found to serve important roles in ribosomal synthesis and function. Individual RPs serve to regulate translation of mRNA molecules, and RPs play a role in cleaving pre-rRNA prior to assembly into ribosomal subunits and exporting pre-subunits from the nucleus into the cytoplasm. Deletion of specific RPs from the genome causes a wide range of problems, such as accumulation of rRNA

in the nucleus, malformed ribosomal subunits, and reduced ability to translate proteins, usually resulting in cell death.[17] Thus, RPs are a worthy subject of study.

While the functional and regulatory role of RPs have only begun to be illuminated in recent decades, even less is known about the role of RP post-translational modifications. The majority of ribosomal PTM publications fall into one of two camps: proteomics studies that seek to identify as many PTMs as possible but pay little attention to their biological roles,[18-20] and biological studies that focus on a single enzyme or PTM at a time, and look for phenotypic differences in mutants that lack the enzyme or PTM.[21-23] While these biological studies occasionally use mass spectrometry to identify known modified proteins or peptides, this is generally done for identification of proteins or PTMs, rather than to quantitate biological changes. Here, we hope to demonstrate that mass spectrometry should be the technology of choice for examining the biological role of ribosomal PTMs, as all of the RPs can be analyzed at once in a quantitative manner from samples grown in a variety of environmental conditions. We also investigate using charge tagging and top-down methods to improve our ability to investigate ribosomal PTMs.[24] Finally, we discuss fluorescence as a quantitative tool to be used in this field of study.[1]

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Figure 1.1

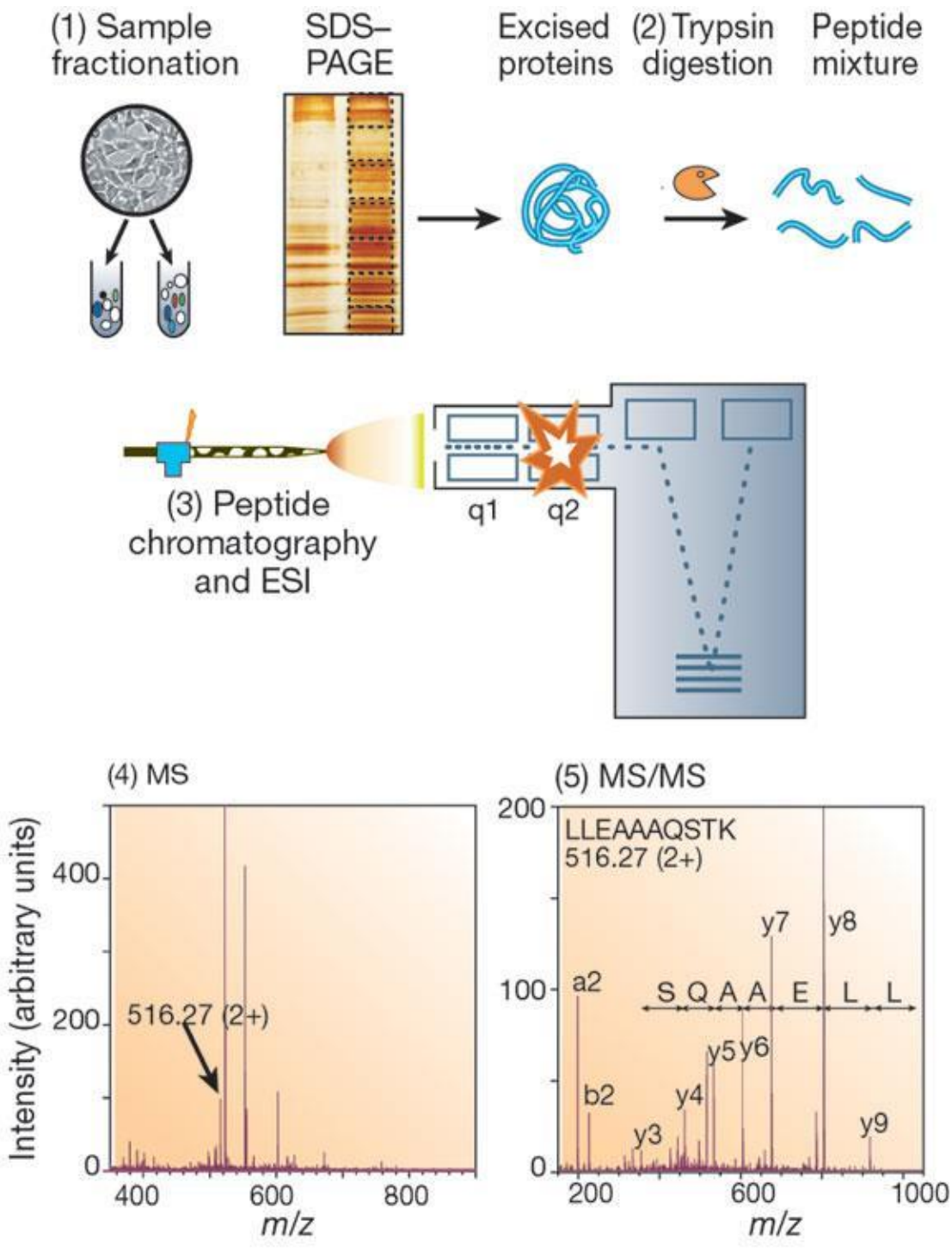

Figure 1.1 - Shotgun Proteomics Workflow, reproduced from Aebersold, 2003. This demonstrates a typical shotgun proteomics experiment, including (1) separation of a complex

protein sample into fractions, (2) digestion of proteins into peptides using the proteolytic enzyme trypsin, (3) transfer into the gas phase by electrospray ionization, (4) analysis by mass spectrometry, and (5) fragmentation by HCD or ETD with the resulting fragments measured in the MS/MS scan.[5]

Figure 1.2 – Ribosome Structure and Biogenesis

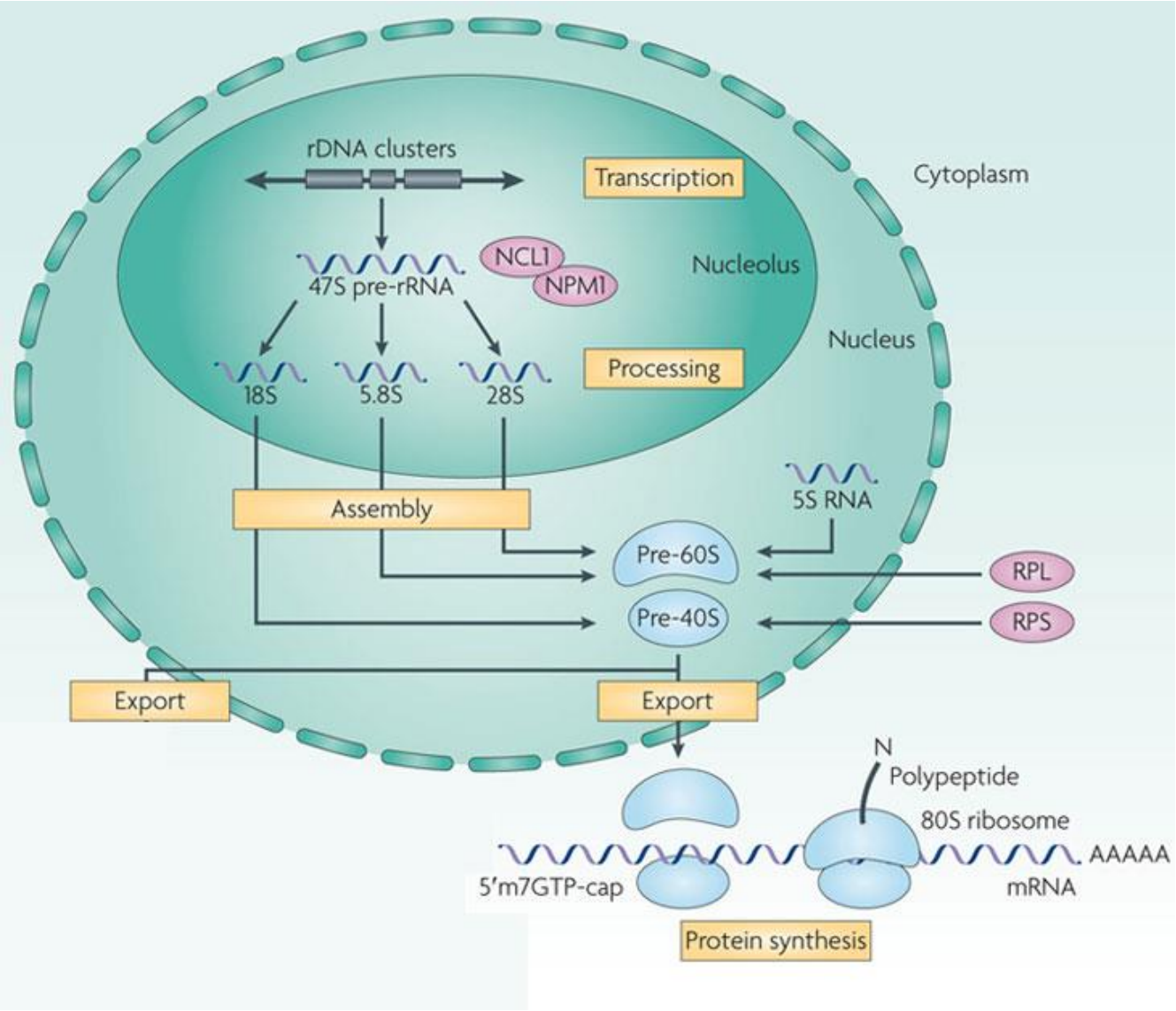

Figure 1.2 – Ribosome biosynthesis, reproduced from van Riggelen 2010. Ribosomal RNA is transcribed in the nucleolus, while ribosomal proteins are translated by ribosomes in the cytoplasm. Ribosomal RNA and ribosomal proteins are transferred to the nucleus to form pre-subunit RNA-protein complexes. The complexes are exported into the cytoplasm, where they complete assembly into the small and large ribosomal subunit.[16]

CHAPTER 2:

MASS SPECTROMETRIC DETERMINATION OF THE EFFECT OF GROWTH PHASE ON YEAST RIBOSOMAL PROTEIN MODIFICATIONS

INTRODUCTION

Ribosomes are complex molecular machines consisting of about 79 proteins and 4 rRNA molecules (in Saccharomyces cerevisiae),[1] and they serve as the center of protein translation in the cell. Protein translation and the synthesis of new ribosomes are energy-intensive processes; ribosome synthesis utilizes 90% of cell energy in yeast during exponential growth.[2] Thus, it is important for the cell to carefully regulate ribosome synthesis and function. Ribosomal regulation has been found to be influenced by a number of environmental conditions, including nutrient availability, environmental stressors and growth phase. Here, we examine regulation of ribosomes by using mass spectrometry to quantify the post-translational modifications (PTMs) on yeast ribosomal proteins under different growth phase conditions.

The yeast population growth curve is generally characterized by three phases: the lag phase, when cells acclimate to a new habitat and population growth is slow; the log phase, when acclimation is complete and exponential growth occurs; and the stationary phase, where population growth stagnates as nutrients grow scarce.[3] As yeast transition from log phase into stationary phase, the cells detect a gradual depletion of resources and begin to enter a quiescent state where they are better able to survive under the starvation conditions.[4] Regulating ribosomal synthesis and function is one component of how cells adapt to environmental stressors. This regulation is an immensely complex process involving changes on multiple "levels" including:

transcription,[5-8] translation,[1, 9] small molecule[10] and protein binding partners for the ribosome,[11] as well as post-translational modifications to ribosomal proteins.[12]

While the regulatory pathways at the transcriptional and translational level have been widely explored, the role of post-translational modifications (PTMs) of the ribosomal proteins has undergone substantially less investigation. Studies of the biological role of ribosomal protein PTMs generally involve creating binding assays or deletion mutants for enzymes known to modify other proteins in the cell, and then observing changes in ribosomal protein PTMs by gel electrophoresis and/or mass spectrometry.[13-23] However, this approach requires prior knowledge of target enzymes and is limited to only identifying drastic PTM changes. One exception to this enzyme-focused trend is a study of phosphorylation of ribosomal protein S6 (RPS6) in quiescent rat fibroblasts, which examined changing environmental conditions rather than enzymatic activity. Radioactive [^{32}P] labeling on a 2D-PAGE gel was used to qualitatively observe that phosphorylation of RPS6 increased in quiescent rat fibroblasts after transfer to new serum.[12] This phosphorylation is hypothesized to encourage translation of mRNAs characterized by a 5' terminal oligopyrimidine, but evidence is elusive.[24] To our knowledge, there has yet to be a quantitative analysis of how ribosomal protein PTMs change under different environmental conditions. Here, we employ mass spectrometry to quantify the dynamic effect of growth phase on ribosomal protein methylation and acetylation.

Mass spectrometry is one of the foremost tools of the field of proteomics. Enabled by the completion of the human genome project, the field of proteomics endeavors to identify all of the protein variants, or proteoforms, found in an organism.[25] Mass spectrometry is often used in combination with on-line liquid chromatography in order to fractionate and identify the components of a complex protein or peptide sample. Mass spectrometry has been used to analyze

ribosomal proteins intact for a top-down study, or after using the proteolytic enzyme trypsin to digest the proteins into peptides for a bottom-up approach. Reverse-phase liquid chromatography is usually coupled to the mass spectrometer, but capillary electrophoresis has also been used successfully.[26] Pre-fractionation methods such as reverse phase chromatography, strong cation exchange, and SDS-PAGE serve to reduce sample complexity to improve the number of peptides or proteins analyzed, at the expense of longer experiment time. Alternatively, antibodies designed to isolate specific subunits or proteins may be used to purify a protein sample, improving data acquired without requiring additional instrument time.

Mass spectrometry is especially useful for elucidating the post-translational modifications of a protein, such as methylation, acetylation, and phosphorylation, which serve to alter or regulate the behavior of a protein. Mass spectrometry-based proteomics has been used previously to analyze ribosomal protein PTMs, although mostly by using the ribosome as a convenient model system for developing proteomics approaches that identify as many proteins and PTMs as possible.[27-34] These proteomics papers have not focused on understanding the role of post-translational modifications on ribosomal proteins. Conversely, biological studies have delved into the role of specific enzymes or PTMs, but have not provided an overview of changes in protein modification. In this study, we combine the strengths of these two approaches to quantify the difference in PTM prevalence on yeast ribosomal proteins in the log phase of cell culture versus those in the stationary phase.

MATERIALS AND METHODS

Yeast Growth

The YIT613 FLAG-tagged ribosome yeast strain was obtained as a generous gift from Professor Toshifumi Inada at Nagoya University, Nagoya, Japan. The strain is mutated to express a FLAG tag (amino acid sequence DYKDDDDK) on ribosomal protein L25. YIT613 strain yeast was grown in YEPD media at 30°C, shaken at 200RPM. Cultures (250 mL) were grown in the presence of 0.0045 g tetracycline and 0.0045 g chloramphenicol, and then transferred into 2 L cultures. Log phase yeast was harvested at OD600 = 0.8, while stationary phase yeast was harvested after incubating for 48 hours. Yeast pellets were washed with lysis buffer (20 mM Tris-HCl, 2 mM magnesium acetate, 100 mM potassium acetate, 100 ug/mL cycloheximide), flash frozen with liquid nitrogen, and stored at -80°C. For this experiment, 3 biological replicates were grown to log phase, and 3 biological replicates were grown to stationary phase.

Ribosome Preparation

Purification of ribosomes was adapted from published reports.[35, 36] One gram of wet yeast cells were resuspended in lysis buffer containing 10 uL/mL HALT protease inhibitor (Thermo Scientific) and 425-600 um acid-washed glass beads (Sigma) and lysed with a Biospec Products Mini-Beadbeater-1. Cell debris was pelleted at 13,000g for 5 minutes and the supernatant lysate was loaded onto a 2.5 mL column of ANTI-FLAG M2 Affinity Gel (Sigma). Bound ribosomes were washed with running buffer (50 mM Tris-HCl, 12 mM magnesium acetate, 100 mM ammonium chloride, 0.02% sodium azide, pH 7.4) and eluted with 100 ug/mL FLAG peptide. Ribosomes were partially concentrated by vacuum evaporation and pelleted by ultracentrifugation using a Type 70 Ti rotor at 50,000 rpm (max 257,000 g) for 3 hours.

Concentrated ribosome samples were stored at -80°C. This procedure was found to yield ~100 ug of ribosomal protein by BCA assay.

Peptide Preparation

Ribosome proteins were isolated using the acetic acid method.[37] Briefly, ribosomes were treated with 66% acetic acid, 0.1M magnesium acetate for 2 hours, ribosomal RNA was pelleted, and ribosomal protein was recovered from the supernatant by precipitation with acetone. Figure 2.1 shows a representative SDS-PAGE analysis of the ribosomal protein preparation. Ribosomal proteins were dissolved in 8M Urea, reduced, alkylated, and digested with Trypsin overnight. Tryptic digests were desalted using Waters Seppak 1cc C18 cartridges, dried under vacuum, and dissolved in 0.1% formic acid.

LCMS

Ten pmol of ribosomal peptide were injected onto a 15 cm long, 100 um ID capillary column packed with Michrom Magic C18 3 um 100A pore resin. A 3 hour 0.1% formic acid/acetonitrile gradient was used to elute peptides into a Thermo LTQ Orbitrap Velos. A data dependent top 10 algorithm was used to isolate peptides with either Higher-Energy Collisional Dissociation (HCD) or Electron Transfer Dissociation (ETD) fragmentation. HCD fragmentation was performed on peptides with 2+ charge or greater at a normalized collision energy of 35%, and ETD was performed on peptides with 3+ charge or greater with an activation time of 50 ms.

Each sample was run in triplicate, for a total of 18 log phase ribosome runs, and 18 saturated ribosome runs.

Data Analysis

MS runs were analyzed individually with Thermo Proteome Discoverer 1.3. Peptides were searched against a S. cerevisiae database from Uniprot (downloaded 5/5/2011). Methylated and acetylated ribosome peptides found within 0.02 Da of the theoretical mass were quantified in Xcalibur Qualbrowser by measuring the extracted ion chromatographic peak area of the unfragmented peptide. Peak areas were normalized using the total peak area of the entire total ion chromatogram. Peak area data were grouped by modified peptide and tested for significance with the student's t-test and the Benjamini-Hochberg correction.[38]

RESULTS AND DISCUSSION

As shown in Figure 2.2, yeast strain YIT613 was grown to either log phase or stationary phase (3 biological replicates each). The ribosomes, which were engineered to express a FLAG tag on RPL25, were isolated by affinity purification. Ribosomal proteins were isolated by using a 66% acetic acid wash to precipitate ribosomal RNA, followed by the addition of four volumes of acetone to precipitate protein. Ribosomal proteins were digested with trypsin and the resulting peptides were separated on a reverse-phase capillary LC column, transferred to the gas phase by electrospray ionization, and analyzed on an LTQ Orbitrap Velos mass spectrometer. Figure 2.3

demonstrates how LC-MS data was analyzed to determine peptide sequence, peptide quantity, and the presence of PTMs.

High purity of the ribosome samples was demonstrated at both the protein and peptide level. Purity of ribosomal proteins prior to tryptic digestion is demonstrated by the SDS-PAGE gel shown in Figure 2.1, which matches similar separations of pure ribosome protein samples as seen in the literature.[36] This coincides with the proteomics results, which demonstrated sample purity on the peptide level. A representative LCMS experiment on log phase ribosomes detected 89 ribosomal proteins, with 7084 out of 7831 peptide spectral matches matching yeast ribosomal peptides (90%). For stationary phase ribosomes, we detected 88 ribosomal proteins with 7061 out of 7901 peptide spectral matches (89%) matching ribosomal peptides. Peptide spectral matches (PSMs) refer to every MS/MS spectrum that can be matched to a peptide, making PSMs the best gauge of sample purity in a quantitative LCMS study. This high quality for both the log phase and stationary phase ribosomes is invaluable for the present quantitative comparison study.

Sample purity was also important for optimizing the number of PTMs observed. On average, LCMS analysis of the FLAG-tag affinity purified ribosomes detected 20 methylated or acetylated peptides per experiment, with 28 modified peptides detected across several replicate experiments (Table 2.1). While ribosomes are regularly seen in proteomic analyses of full yeast digests, a search for methylated and acetylated ribosomal peptides in a full yeast digest experiment yielded only 8 modified peptides on average (data not shown). The most common method to improve LCMS data is off-line fractionation of yeast digest peptides prior to LCMS analysis, a process commonly referred to as pre-fractionation. For example, off-line high-pH reverse phase chromatographic separation of yeast digest peptides into eight fractions was found to improve the number of methylated or acetylated peptides identified to 24, at the cost of eight

times the instrument analysis time. (Note that no phosphoryl groups were identified, as phosphorylation studies usually require a phosphopeptide enrichment step to improve the number of phosphopeptides detected at the expense of all other peptides.)[39] Thus, utilizing a FLAG-tag affinity purification allowed us to improve the quality of our data while shortening required instrument time, allowing for analysis of multiple biological and technical replicates.

To further expand the range of peptides detected, we analyzed each sample using both Higher-Energy Collisional Dissociation (HCD) and Electron Transfer Dissociation (ETD) fragmentation, which have been found to produce complementary data.[40] As shown in Table 2.1, 11 modified peptides were only detected using HCD, while 3 peptides were detected only using ETD. 14 peptides were detected using both methods, allowing us to combine their data as technical replicates. Thus, while ETD fragmentation allowed us to detect a few additional modified peptides, it may not be worth the additional instrument time in future experiments.

Quantitative data showing the relative amounts of modified ribosomal peptides in log-phase versus stationary phase yeast is also shown in Table 2.1. Modified peptides were quantified by constructing the extracted ion chromatogram of the most intense isotopic peak in the full scan and then measuring the peak area (Figure 2.3). While spectral counting is commonly used for label-free quantification of proteins, our goal was to quantify PTM prevalence on the peptide level. Note that peak area has been shown to be as reliable a label-free peptide quantification method as spectral counting is for protein quantification.[41] Peak areas were normalized by total ion chromatographic peak area to compensate for differences in injected sample mass. When comparing the log phase to the saturated phase, some PTMs were seen to increase in abundance, while others decreased. Most changes were minor, ranging from 1

to 1.5-fold, but occasionally larger changes were seen, including a 10-fold increase for the dimethylated rGGFGGR peptide in the stationary phase.

Statistically significant changes in modified peptide quantity between log phase and stationary phase samples were found using the student's t-test[41] with the Benjamini-Hochberg correction.[38] Two peptides met the p-value cutoff of 0.05: a 1.8-fold decrease in methylation for k(Me)VSGFKDEVLETV of RPS1B, and a 10-fold increase in dimethylation for r(DiMe)GGFGGR of RPS2. We will henceforth refer to these modifications as RPS1B K243 methylation and RPS2 R10 (di)methylation, for the amino acid position in the protein. While we did not find published reports in the literature on the biological role of RPS1B K243 methylation, RPS2 R10 dimethylation is a known modification, and we will now discuss what is known about its biological roles.

We have used mass spectrometry to look for quantitative differences in methylation and acetylation of S. cerevisiae ribosomal proteins. Of the 27 modified peptides we saw, we found RPS2 R10 dimethylation to be more prevalent in the stationary phase, and RPS1B 243K methylation to be less prevalent. It is interesting to see that only methylation was found to depend on growth phase, as methylation has been a recent focus of studies of ribosome regulation.[13, 15, 16, 18-23] RPS2 R10 dimethylation is of particular note, in that it is 10-fold more prevalent in the stationary phase with high confidence. Lipson et al. informally observed that RPS2 methylation varied depending on growth conditions, although they did not publish this data.[22]

Methyltransferase studies have identified Rmt3 in S. pombe, Rmt1 in S. cerevisiae, and Prmt3 in mouse as catalysts for RPS2 R10 methylation. Rmt3-null S. pombe mutants exhibit an

imbalance in ribosomal subunits due to underproduction of the small subunit and increased ribosomal density on mRNAs encoding 40S ribosomal proteins. Adding methylated RPS2 mitigates this imbalance, suggesting that RPS2 arginine methylation regulates small subunit production. However, later studies found that Rmt3 mutants that were rendered catalytically inactive yet still able to bind RPS2 protein also exhibit normal ribosome production. Furthermore, replacing RPS2 methylarginines with lysines also does not perturb ribosomal subunit balance. Thus, Rmt3 was found to exhibit a methyltransferase-independent function while methylation of RPS2 arginines serve a secondary role. Rmt3 is hypothesized to act as a gauge to see how much free RPS2 is available, and perhaps control degradation of free RPS2 or intact small subunits by the proteasome.[20, 42-44]

In mammalian cells, deleting the enzyme Prmt3 causes hypomethylation of RPS2. Mice lacking Prmt3 exhibit smaller embryos during gestation, but ribosome stability and function are not affected.[21] However, it has been suggested that when Prmt3 binds RPS2, it provides resistance to ubiquitination and subsequent degradation by the proteasome.[45]

In S. cerevisiae, deletion of Rmt1 causes no phenotypic difference in ribosome stability. Rmt1 is known to bind and/or methylate a number of proteins that process and export mRNA. Thus, methylation of RPS2 may play a role in exporting rRNA to the cytosol.[22, 23] This is consistent with RPS2's known functions of processing and exporting 20S rRNA, a precursor for 40s ribosomal subunits. In S. pombe, depletion of RPS2 causes a delay in 20S rRNA cleavage at the A2 site, causing underproduction of 18S rRNA.[43] S. cerevisiae mutants deficient in RPS2 show normal levels of 20S rRNA production in the nucleus but reduced levels of 20S rRNA reaching the cytoplasm, proving that RPS2 plays a role in exporting 20S rRNA from the nucleus to the cytoplasm (Ferreira-Cerca). Furthermore, methylation of Rmt1 target proteins such as

Npl3, Hrp1, and Nab2 promotes their dissociation from nuclear factors and facilitates their export from the nucleus.[46-48] Thus, it stands to reason that methylation of RPS2 plays a role in processing and export of 20S rRNA from the nucleus.

Our observation that RPS2 R10 dimethylation increases tenfold in stationary phase suggests that the cell may be regulating the ability of RPS2 to process and export 20S rRNA, thus regulating 40S ribosomal subunit production as a response to the depletion of available nutrients. A second, albeit less likely, possibility is that the number of methylated RPS2 proteins is constant while fewer ribosomal proteins are being synthesized by the cell. As a constant weight of ribosomal protein is injected into the mass spectrometer for each experiment, our observations may be due to a higher proportion of methylated RPS2 in the stationary phase yeast while the actual number of methylated RPS2 proteins per cell remains the same. This explanation requires two conditions: that the methyltransferase activity of Rmt1 is constant in both growth phases, and that unmodified RPS2 is selectively degraded by the proteasome. This scenario fits a hypothesized role of RPS2 methylation in S. pombe, where the interaction between methyltransferase Rmt3 and RPS2 serves to gauge the amount of RPS2 that is incorporated into pre-40S subunits. However, data that proves the viability of this hypothesis has not been published. Testing which of these possibilities is the case would require an adjustment in the ribosome isolation procedure. As described in our methods section, 1g of yeast cells per sample is run through the 2.5 mL anti-FLAG column in order to saturate the column with ribosomes. While this maximizes the ribosome yield of each sample preparation, the ability to quantify an absolute mass of ribosome proteins is lost. To achieve absolute quantification, a sample would need to be prepared from ≤0.2g yeast cells so that the number of ribosomes in the crude lysate does not saturate the anti-FLAG column. The total ribosome sample would then be

analyzed for protein content by either BCA assay or SDS-PAGE gel staining. In combination with the proportional measurements made by mass spectrometry, this total protein mass would allow absolute quantification of the number of methylated RPS2 proteins per cell. Alternatively, a sucrose gradient purification could be used to avoid the mass-limiting anti-FLAG step altogether.

CONCLUSIONS

We have demonstrated the use of mass spectrometry to quantitatively compare the prevalence of ribosomal post-translational modifications among yeast at different phases of growth. Our observations of RPS2 R10 dimethylation increasing 10-fold in stationary phase yeast provide new evidence for the role of RPS2 methylation in 20S rRNA processing and export; this coincides with biological studies in the literature, lending credence to the biological relevance of this study. To our knowledge, the observation of RPS1B 243 methylation decreasing in stationary phase yeast has not been reported, suggesting a novel role for RPS1B methylation that invites further investigation. While we were only able to illuminate a small piece of the ribosome PTM puzzle, our work demonstrates the utility of mass spectrometry for quantifying dynamic changes in PTMs.

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Table 2.1

Protein	Peptide Sequence	Modifications	Fragment	Ratio	P Value
S2	**rGGFGGR**	**R1(Dimethyl)**	**HCD/ETD**	**10.546**	**0.0003291**
S1-B	**kVSGFKDEVLETV**	**N-Term(Methyl)**	**HCD/ETD**	**0.5326**	**0.0005005**
L7-A	TAEQVAAEr	R9(Methyl)	HCD	0.7529	0.0069372
S1-B	vSGFKDEVLETV	N-Term(Methyl)	HCD	0.7388	0.0506797
L8-A	KMGVPYAIVk	K10(Methyl)	HCD/ETD	0.7826	0.0568703
L12	IQNrQAAASVVPSASSLVITALK	R4(Methyl)	HCD/ETD	1.4078	0.0805102
S21-A	aIPGEYVTYALSGYVR	N-Term(Methyl)	HCD/ETD	0.7124	0.0982029
L9-A	YIQTEQQIEVPEGVTVSIk	K19(Methyl)	HCD	1.7838	0.1165891
L33-A	IEGVATPQDAQFYLGk	K16(Methyl)	HCD/ETD	0.8251	0.1211994
S29-B	VCSSHTGlVrK	C2(c); R10(Methyl)	HCD	0.5375	0.1282221
S21-A+B	mENDKGQLVELYVPR	N-Term(Acetyl)	HCD/ETD	0.7858	0.1530007
L42	KQSGFGGQTkPVFHK	K10(Methyl)	HCD/ETD	0.6982	0.1846366
L33-A	IEGVATPQDAQFYLGkR	K16(Methyl)	HCD	1.3066	0.2736336
L16-A	vFEGIPPPYDKK	N-Term(Methyl)	HCD/ETD	0.7726	0.2793004
L42	KQSGFGGQTkPVFHKK	K10(Methyl)	ETD	0.6455	0.284468
L23	DGVFLYFEDNAGVIANPkGEMkGsAITGPVGK	K18(Dimethyl); K22(Dimethyl)	HCD	2.5974	0.3172123
L42	QSGFGGQTkPVFHKK	K9(Methyl)	HCD/ETD	0.6603	0.3592812
L31-A	LHMGTDDVr	R9(Methyl)	HCD	0.9015	0.3819633
S8	KNVkEEETVAK	K4(Acetyl)	ETD	0.6461	0.3996989
L42	QSGFGGQTkPVFHK	K9(Methyl)	HCD/ETD	0.6962	0.4622182
L24-B	MkVEVDSFSGAK	K2(Methyl)	HCD/ETD	0.8909	0.5313079
L16-A	lKVFEGIPPPYDK	N-Term(Methyl)	ETD	0.9058	0.5475184
L16-A	vFEGIPPPYDK	N-Term(Methyl)	HCD	0.9541	0.6081701
L16-A	lKVFEGIPPPYDKK	N-Term(Methyl)	HCD/ETD	0.9093	0.6444318
L42	ASLFAQGkR	K8(Methyl)	HCD/ETD	0.8624	0.8020471
S21-A+B	MENDkGQLVELYVPRK	M1(Oxidation); K5(Acetyl)	HCD	0.905	0.8058914
S29-B	VCSSHTGlVr	C2(c); R10(Methyl)	HCD	0.9286	0.8302865
S28-A	mDNKTPVTLAK	N-Term(Acetyl)	HCD	1.014	0.9499086
S21-A+B	mENDKGQLVELYVPRK	N-Term(Acetyl)	HCD	0.9976	0.9785785

Table 2.1. Methylated and acetylated ribosomal peptides detected by mass spectrometry. Peptides were fragmented with HCD and/or ETD fragmentation, and then searched against a Uniprot *S. cerevisiae* database. 29 modified peptides were identified and quantified by extracted ion chromatogram (XIC) peak area across all biological and technical replicates. The average peak areas were used to find the stationary phase/log phase ratio, and tested for statistical significance with the student's t-test. Resulting p-values were adjusted with the Benjamini-Hochberg correction, and two peptides, RGGFGGR and KVSGFKDEVLETV were found to exhibit statistically significant changes in modified peptide quantity.

Figure 2.1

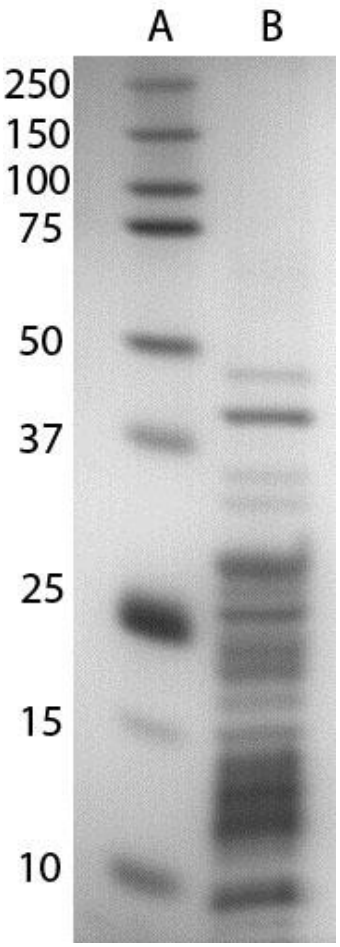

Figure 2.1. SDS-PAGE gel of *Saccharomyces cerevisiae* ribosomal proteins. Lane A contains molecular weight markers. Lane B contains ribosomal proteins after acetic acid wash, acetone precipitation, and resuspension in SDS-PAGE buffer. Multiple proteins are visible, ranging from 5 kDa to 45 kDa.

Figure 2.2

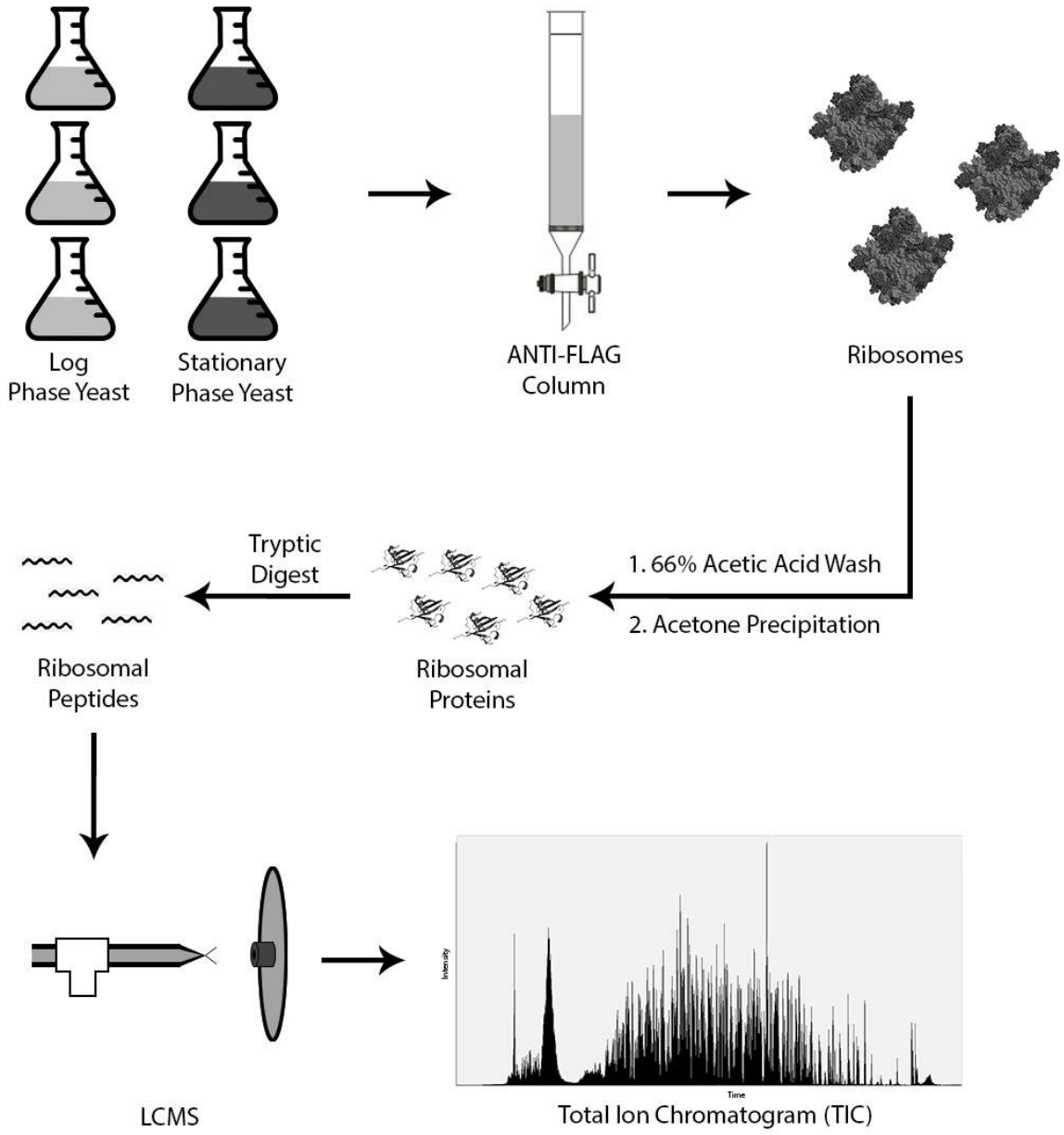

Figure 2.2. Experimental workflow. Three replicates each of YIT613 strain yeast were grown to log phase and stationary phase. Yeast were lysed by bead beating and loaded onto an ANTI-FLAG column. FLAG-tagged ribosomes were retained on the column and eluted with FLAG peptide. Ribosomal RNA was precipitated in 66% Acetic acid and 0.1M magnesium acetate and

dissolved ribosomal proteins were collected by acetone precipitation. Ribosomal proteins were resuspended in 8M urea and digested with the proteolytic enzyme trypsin, and the resulting tryptic peptides were analyzed by LCMS.

Figure 2.3

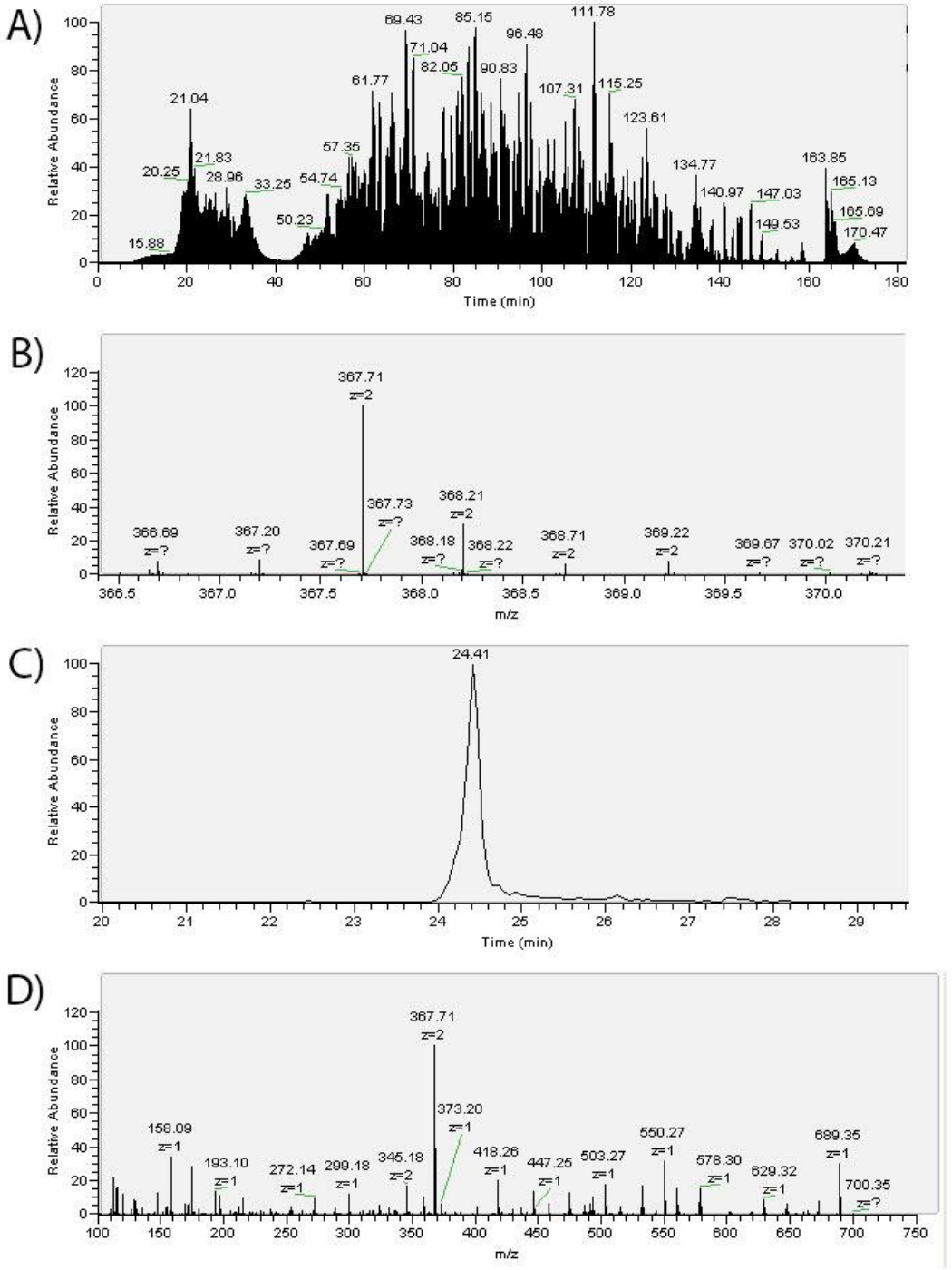
A)
Relative Abundance
Time (min)
15.88
20.25
21.04
21.83
28.96
33.25
50.23
54.74
57.35
61.77
69.43
71.04
82.05
85.15
90.83
96.48
107.31
111.78
115.25
123.61
134.77
140.97
147.03
149.53
163.85
165.13
165.69
170.47
B)
Relative Abundance
m/z
366.69 z=?
367.20 z=?
367.69 z=?
367.71 z=2
367.73 z=?
368.18 z=?
368.21 z=2
368.22 z=?
368.71 z=2
369.22 z=2
369.67 z=?
370.02 z=?
370.21 z=?
C)
Relative Abundance
Time (min)
24.41
D)
Relative Abundance
m/z
158.09 z=1
193.10 z=1
272.14 z=1
299.18 z=1
345.18 z=2
367.71 z=2
373.20 z=1
418.26 z=1
447.25 z=1
503.27 z=1
550.27 z=1
578.30 z=1
629.32 z=1
689.35 z=1
700.35 z=?

Figure 2.3. A representative LCMS experiment showing data for dimethylated peptide rGGFGGR. In Fig. 2.3A, the Total Ion Chromatogram (TIC) represents all of the yeast ribosomal peptides detected by the mass spectrometer. Several full scan mass spectra are taken per second, each of which may detect multiple peptides. Fig. 2.3B shows the isotopic distribution of the +2 charge state for rGGFGGR as seen in a full scan mass spectrum. In Fig. 2.3C, the Extracted Ion Chromatogram (XIC) corresponds to the signal intensity over time of the 367.71 m/z ion, the most intense isotopic peak for rGGFGGR. The peak area is integrated to yield a quantitative measure of the amount of peptide in the sample. Finally in Fig. 2.3D, the 367.71 m/z ion was isolated for HCD fragmentation and the MS/MS spectrum is shown. The peptide is identified by comparison to a Uniprot S. cerevisiae database, and characteristic fragmentation peaks are checked for mass shifts to identify PTMs. A mass shift of +42.01 indicates the presence of an acetyl group, while a +14.02 or +28.03 mass shift corresponds to a methyl or dimethyl group, respectively. Trimethylations (+42.04) were not detected.

CHAPTER 3:

ENHANCING ETD FRAGMENTATION OF RIBOSOMAL PEPTIDES BY CHEMICAL DERIVATION

INTRODUCTION

Mass spectrometry is the workhorse of proteomics. The bottom-up approach has proven to be the standard proteomics experiment due to its robustness and ease of use. However, existing bottom-up methods can only identify a subset of the peptides ionized in a single experiment, which limits sequence coverage and the number of identified proteoforms (e.g., isoforms from alternative splicing, protein variants arising from genetic variation, and/or proteins with various post-translational modifications (PTMs)). Thus, there is still a need to increase the number of peptides identified in a single bottom-up experiment.[1] Several different methods are commonly employed by the proteomics community to alleviate this problem, including pre-fractionation, longer chromatographic gradients, customized proteomic databases, and peptide derivatization strategies to improve chromatography and ionization. Here, we discuss the use of chemical derivation to improve ETD fragmentation for ribosomal peptides, with the goal of increasing sequence coverage and consequently, the number of PTMs identified.

It is well known that ETD is particularly ineffective for the dissociation of peptide dications ($z = 2$).[2-5] As a result, it is common to pair ETD-MS with an alternative enzyme such as endoproteinase LysC instead of trypsin, as LysC gives rise to longer peptides with correspondingly larger charge states. However, LysC still produces more peptides with $z = 2$ (40% of the total) than any other charge state,[6] and even though LysC produces more peptides

with charge states of z > 2, not all such peptides are equivalent in their propensity to yield a quality fragmentation spectrum. We have shown that the percent fragmentation and probability of identification, following ETD, correlates inversely with the residues/charge ratio, which can be approximated by the precursor m/z.[4] The reason behind this relationship is as follows. A precursor having a lower residue/charge ratio, or m/z, has a higher charge density, which causes increased repulsion between the *c*- and *z•-type* fragment ions created by backbone cleavage after electron transfer. When Coulombic repulsion overcomes the non-covalent interactions between the two fragments, they separate and are detected at their respective m/z values. If the charge density is too low, the peptide fragments fail to separate after electron-induced bond cleavage, and only the charge-reduced precursor m/z value is observed, instead of the desired fragmentation products. This phenomenon is referred to as electron transfer without dissociation, or ETnoD.[7, 8]

In order to enhance dissociation of the *c*- and *z•-type* fragment ions, a number of different approaches have been developed. Collisional[9] and IR activation[10] have been used to increase the internal energy of peptides in low charge states. The added thermal energy "activates" the peptide, making fragmentation more favorable. Alternatively, the charge states of peptides and proteins can be increased prior to fragmentation, either by changing the electrospray conditions with solvent additives,[11-13] or by chemical derivatization. The most common derivatization reactions use N-hydroxysuccinimide (NHS) esters to modify amine function groups, causing them to target the n-termini and lysine side-chains of peptides and proteins. This eliminates native protonation sites, but can be counteracted by designing the modifying reagent with a replacement charging site or a fixed positive charge charge.[14-21] Alkylation of peptide amino groups with aldehydes is another common derivatization, with the added benefit of preserving

charge sites.[21-29] The thiol group of cysteine residues has also been derivatized,[30-34] and this strategy has been evaluated for ETD enhancement.[35, 36] Another approach added fixed charges to methionine residues by converting the thioether moiety into sulfonium groups.[37] Finally, carboxyl groups on peptides have been modified and tested for ETD fragmentation enhancement (Xu et al.; Zhang et al.; Qiao et al.; Zhang, Al-Eryani and Ball). Carboxyl groups are neutral during typical positive-mode electrospray ionization, making them a prime candidate for the addition of fixed-positive charge tags. Unfortunately, previous carboxyl derivatization reactions have suffered from low reaction efficiency.[38]

Completeness of the labeling reaction is especially important for complex samples. Low reaction efficiency results in unmodified or partially modified peptides, each of which have different retention times and masses from the modified form. Thus, an incomplete reaction can easily double or triple sample complexity, a significant hindrance to analysis of complex samples where instrument bandwidth already limits peptide identifications. Furthermore, the addition of too many dynamic modifications during database searching increases the chance of false peptide identifications. Fortunately, Krusemark, et al. of the Smith lab recently published a two-step method that yields nearly complete derivatization of all carboxyl groups on small proteins or peptides.[28] The first step protects native amino groups by methylating them with formaldehyde, a reaction widely used in proteomics for multiple purposes because the methylated amino sites still carry a positive charge during positive-mode electrospray ionization. The second step is an amidation reaction whereby the peptide carboxylic acids are converted to tertiary or quaternary amine groups, producing substantially higher charge states in the gas phase. We chose to take advantage of this efficient derivatization method to enhance ETD fragmentation of ribosomal peptides with the goal of increasing the number of post-translational modifications (PTMs)

identified. C3-methylTert amidation proved more effective at improving sequence coverage and number of peptide spectral matches (PSMs), while C4-methylTert amidation proved more successful at identifying new acetylated peptides.

MATERIALS AND METHODS

Reagents and Materials.

(7-Azabenzotriazol-1-yloxy)tripyrrolidinophosphonium hexafluorophosphate (PyAOP) was purchased from Applied Biosystems (Foster City, Ca, USA). 3-(dimethylamino)-1-propylamine (C3-methylTert) was purchased as a free-base from Sigma-Aldrich. 4-(dimethylamino)-1-butylamine (C4-methylTert) was received as a gift from Prof. Shane Lamos at St. Michael's College in Vermont.. C3-methylTert and C4-methylTert were each converted to their di-hydrochloride salts by the drop-wise addition of a stoichiometric amount of 1 M HCl in ether while stirring in an ice bath. The ether solvent was removed from the solid products by rotary evaporation, and then acetone was added and subsequently removed by rotary evaporation.

Yeast Ribosome Purification

Yeast ribosomes were purified as described in Chapter 2. Briefly, the YIT613 strain of *Saccharomyces cerevisiae* was grown in YPD media to an OD600 of 0.8. Yeast cells were lysed by bead beating and lysate was loaded onto a 2.5 mL ANTI-FLAG M2 Affinity Gel column. FLAG-tagged ribosomes were eluted using FLAG peptide, concentrated by ultracentrifugation. Ribosomal proteins were isolated by 66% acetic acid wash and acetone precipitation.

Protein Digestion and Peptide Derivitization

Precipitated ribosome proteins were dissolved in 8M urea, reduced with DTT, alkylated with iodoacetamide, and digested with trypsin overnight at 37°C at an enzyme:protein ratio of 1:20. The tryptic peptides were desalted using a Waters Seppak 1cc C18 cartridge and dried under vacuum.

100 ug ribosomal peptides were dissolved in 50:50 methanol:H_2O to a concentration of 2 mg/mL. 20 ug were stored as an underivatized control sample, and the remaining 80 ug peptides were treated with 40 mM formaldehyde (HCHO), 60 mM borane-pyridine complex, and 50 mM 4-methylmorpholine (NMM) for 2 hours at room temperature. The resulting dimethylated peptide mixture was dried by vacuum centrifugation and redissolved in wet dimethyl sulfoxide (DMSO) to a concentration of 1.5 mg/mL. The dimethylated peptide solution was treated with a solution of 30 umol of C3-methylTert dihydrochloride salt, 2.6 uL NMM, 5 uL water for a final concentration of 450 mM amine and 350 mM NMM. After mixing, solid PyAOP was added to a concentration of 60 mM, and the mixture was allowed to react for 2 hours at room temperature, after which the reaction was quenched by adding 0.1% formic acid so as to dilute the DMSO to 5% (v/v). The reaction mixture was extracted with two 1 mL chloroform washes to remove reaction by-products, and the aqueous layer was dried by vacuum centrifugation. The dried peptides were resuspended in 0.1% TFA, desalted using a Waters Seppak 1cc C18 cartridge and dried again under vacuum.

Mass Spectrometry and Data Analysis

Ribosomal peptides were analyzed as underivitized peptides, dimethylated peptides, and fully derivitized peptides that had undergone both dimethylation and C3-methylTert labeling. Peptides were dissolved in 0.1% formic acid, separated on a 100 um ID capillary column packed with Michrom Magic C18 3 um 100A pore resin, and analyzed on a Thermo LTQ Orbitrap Velos.

RESULTS AND DISCUSSION

Ribosomal peptide samples were prepared as discussed in Chapter 2. Chemical derivitization of peptides was conducted in two steps. First, peptide amino groups were dimethylated using formaldehyde, borane-pyrimidine, and NMM. Next, peptide carboxyl groups were amidated using NMM, PyAOP, and a specially prepared amine salt. Ribosomal peptides were amidated with both C3-methylTert and C4-methylTert amines to determine which provided the greatest improvement to ETD fragmentation. Both steps of the peptide derivitization reaction were found to be highly efficient. The efficiency of amino dimethylation has been shown by prior studies to be essentially complete.[22, 26-28] We found that ribosomal peptides were dimethylated just as efficiently. Carboxyl amidation of ribosomal peptides was also found to be very efficient, with a minimum estimated reaction efficiency of 94.38% for C3-methylTert amidation as shown in Table 3.1.

C3-methylTert modified and C4-methylTert modified ribosome peptides were analyzed by LCMS, along with a separate unmodified control sample for each modified sample. Peptides were fragmented by ETD and the resulting MS/MS data was searched against a yeast database downloaded from Uniprot. While the yeast ribosome is generally said to contain about 80 proteins, the Uniprot database we used contained 127 protein entries due to closely related

protein isoforms. Due to the purity of our ribosomal samples, greater than 90% of the peptide spectral matches (PSMs) arose from 40s and 60s ribosomal proteins. Consequently, we chose to summarize the data for only these ribosomal proteins, rather than also including the data from the other proteins that had much lower abundance and were not the focus of the sample preparation.

Compared to unmodified peptides, C3-methylTert labeling only slightly increased the number of proteins seen, but increased the number of peptides seen by 58% and the number of PSMs seen by 115 % (Fig 3.2). However, C4-methylTert labeling saw a slight decrease in the number of proteins seen and the number of peptides seen, and a slight increase in the number of PSMs seen. As ribosomal peptides are very basic, tryptic digestion gives rise to significantly hydrophilic peptides, some of which may not bind well to a C18 reverse phase column. Theoretically, C4-methylTert amines should be more suitable than C3-methylTert amines for diminishing this hydrophilicity, and thus allow detection of more peptides that would normally be too hydrophilic to resolve on a reverse phase column. However, the opposite seems to be the case, possibly because of the lower purity of the C4-methylTert amine salt used.

With the improvement in sequence coverage resulting from C3-methylTert amidation, we were interested to see if any new acetylated peptides were detected. As shown in Fig 3.3, 5 acetylated peptides were identified in the unmodified sample, while 3 acetylated peptides were found in the C3-methylTert amidated sample, with 1 new acetylated peptide detected. It should be noted that naturally occurring methyl groups could not be distinguished from methyl groups produced by the formaldehyde reaction, making acetylation the focus of this study. Interestingly, 4 acetylated peptides were found in the unmodified sample, while 3 entirely different acetylated peptides were found in the C4-methylTert amidated sample. It is notable that despite the lack of improvement in number of peptides seen, the C4-methylTert sample appears to have produced

data that is complementary to the unmodified peptide data, allowing for the detection of new acetylated peptides. This suggests that longer amine chains may be beneficial for reducing hydrophilicity of ribosomal peptides as theorized.

It is possible that we may observe even greater improvement in acetylated peptide identification if we improve the purity of the amine salt reagents. Additional extraction or chromatography steps after synthesis of the amine hydrochloride salt may help to remove remaining by-product. With a more optimized purification method, perhaps this experiment may be more efficiently repeated in the future, with the inclusion of C5-methylTert amidation as well. Other future improvements to the experimental method involve the use of isotopically pure reagents. "Heavy" formaldehyde containing deuterium atoms can be used for the methylation reaction to create methyl groups of mass 18 or amu as opposed to the naturally-occurring 15 amu, allowing us to identify naturally-occurring methylation PTMs.[39] Isotopically-pure amines consisting of only ^{13}C or ^{12}C could be used to amidate different biological samples in a manner similar to ICAT quantification.[30] Essentially, creating "heavy labeled" and "light labeled" samples allow for both samples to be mixed and analyzed together. Heavy and light versions of each peptide co-elute and ionize with the same efficiency, but are detected at different m/z values in the mass spectrum. Thus, combining our derivatization method with existing isotopic labeling methods will allow enhanced ETD fragmentation and isotopically-labeled quantification to be performed simultaneously.

CONCLUSIONS

In this work, we discussed the use of an efficient derivatization reaction to enhance ETD fragmentation for ribosomal peptides. While C3-methylTert amidation provided greater improvement for the number of ribosomal PSMs, C4-methylTert amidation provided acetylated peptide identifications that were complementary to those detected by analysis of underivatized peptides. We believe this demonstrates that amine reagents with longer aliphatic chains provide greater improvement to peptide chromatographic resolution of the hydrophilic ribosomal peptides. While peptide derivatization increased the number of acetylated peptide identifications, we concluded that it was not significant enough an improvement to merit use when quantifying the effect of growth phase on ribosomal protein PTMs. In the future, we will work to improve the purity of our synthesized amine hydrochloride salts and employ heavy isotope reagents to identify naturally-occurring methylation and perform relative quantification of two biological samples. These method improvements will further increase the utility of this peptide derivatization strategy.

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Table 3.1

Protein	Peptide	Modifications	Intensity	% Efficiency
S1A	FDVGALMALHGEGSGEEK	2 Dimethyl, 5 C3Tert	3.46E+06	97.3651192
		2 Dimethyl, 4 C3Tert	5.25E+05	
L7A	QANNFLWPFK	2 Dimethyl, 1 C3Tert	1.37E+05	62.59
		2 Dimethyl	8.19E+04	
S2	GYWGTNLGQPHSLATK	2 Dimethyl 1 C3Tert	7.73E+06	83.30
		2 Dimethyl	1.55E+06	
S2	AVVVVGDSNGHVGLGIK	2 Dimethyl, 2 C3Tert	6.56E+05	95.14796972
		2 Dimethyl, 1 C3tert	7.05E+04	
S13	KGLTPSQIGVLLR	2 Dimethyl, 1 C3Tert	2.80E+06	95.73
		2 Dimethyl	1.25E+05	
L7A	LIEPYVAYGYPSYSTIR	1 Dimethyl, 2 C3Tert	4.87E+06	99.28251938
		1 Dimethyl, 1 C3Tert	7.09E+04	
L8B	LKVPPTIAQFQYTLDR	2 Dimethyl, 2 C3tert	1.22E+06	98.17564366
		2 Dimethyl, 1 C3tert	4.62E+04	
L8B	YGLNHVVSLIENK	2 Dimethyl, 2 C3tert	5.76E+04	98.17564366
		2 Dimethyl, 1 C3tert	2.12E+06	

Table 3.1. Efficiency of C3-methylTert carboxyl amidation reaction for ribosomal peptides. Eight peptides for which fully reacted and partially reacted peptides were detected were quantified by signal intensity. Average efficiency among these eight peptides was calculated to be 94.38%. Note: as only partially reacted peptides were chosen, the actual average reaction efficiency is higher. Thus, 94.38% is considered to be the “minimum reaction efficiency.”

Figure 3.1

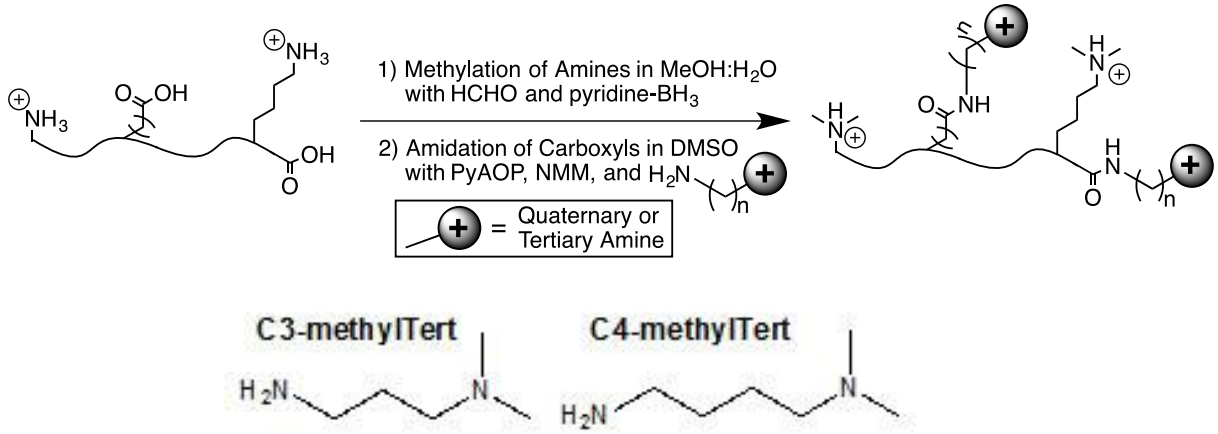

Figure 3.1. Labeling strategy to increase peptide charge states. First, the native primary amine groups in the peptides are dimethylated to prevent them from reacting in the second step. Then, the carboxylic acid groups are amidated with a labeling reagent having a primary amine on one end and a tertiary amine on the other end. The structures and abbreviated names are shown for the two labeling reagents employed in this work. (All chemical reagent abbreviations are defined in the Experimental section).

Figure 3.2

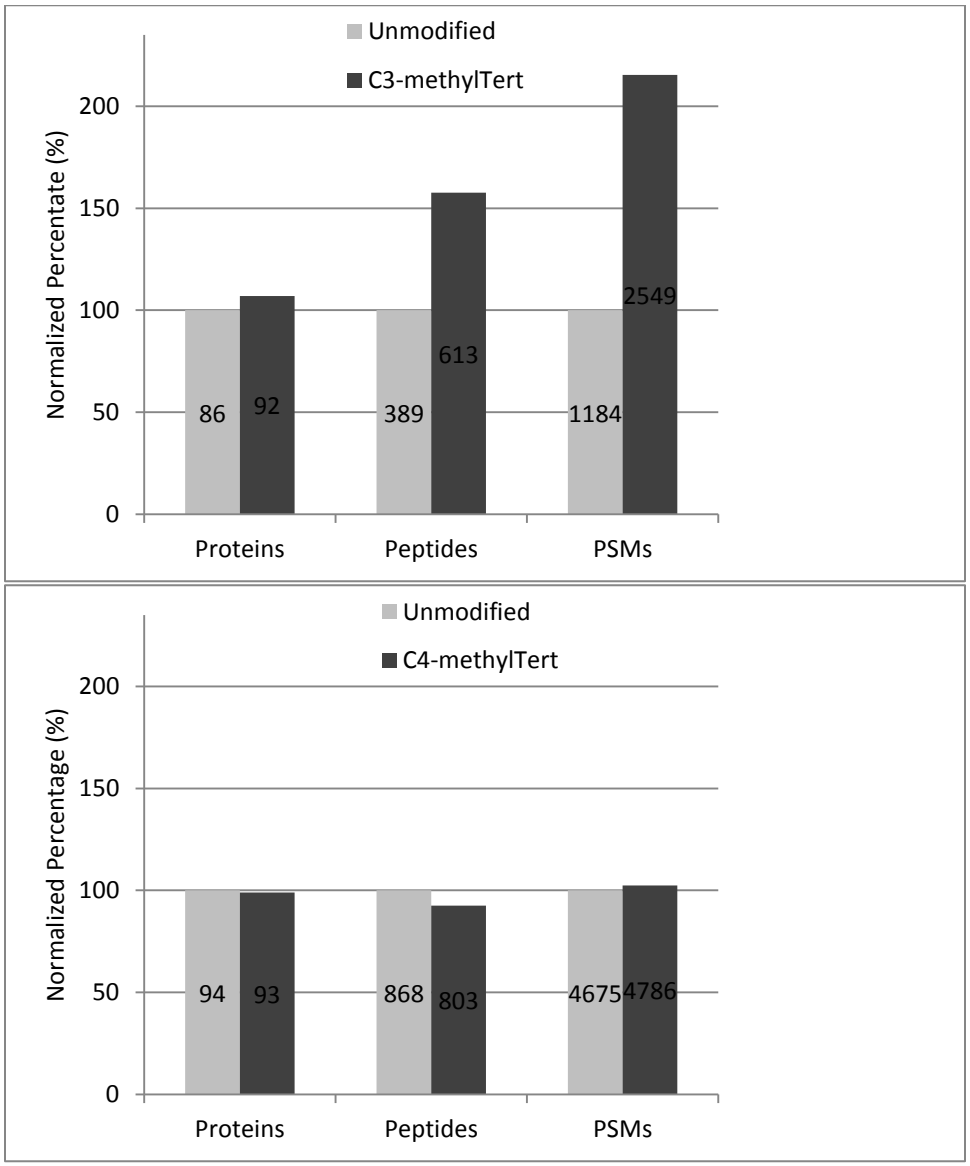

Figure 3.2. ETD-MS results for unmodified versus C3-methylTert amidated peptides, and unmodified versus C4-methylTert amidated peptides. C3-methylTert amidation of ribosome peptides significantly increase PSMs.

Figure 3.3.

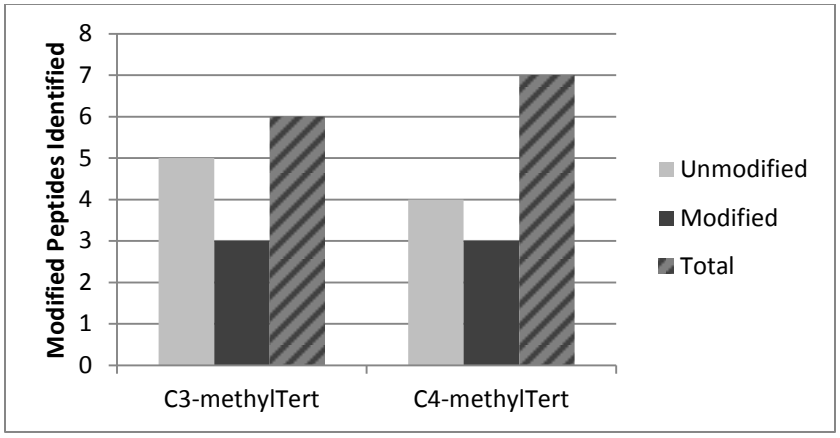

Figure 3.3. Acetylated peptides identified for unmodified versus C3-methylTert amidated peptides, and unmodified versus C4-methylTert amidated peptides. Analysis of unmodified peptides in combination with C4-methylTert amidated peptides yields the most acetylated peptide identifications.

CHAPTER 4:

GELFREE PREFRACTIONATION AND TOP-DOWN MASS SPECTROMETRY OF RIBOSOMAL PROTEINS

INTRODUCTION

Mass spectrometry is the foremost tool for the field of proteomics. However, most proteomics studies use a bottom up approach, where proteins are enzymatically digested into peptides. While this strategy is useful for identifying proteins and individual post translational modifications (PTMs), contextual information about the entire protein is lost. For example, two PTMs may be identified for the same protein sequence, but it is unknown if the two modifications originated from the same individual protein or from different forms of the protein, called proteoforms. The term "proteoform" has been proposed to mean the different forms in which a given protein of interest can be found, including variation from alternative splicing of the RNA transcript, allelic variation leading to codon substitutions, and post-translational modifications.[1] In order to identify different proteoforms, top-down proteomics methods have been developed where intact proteins are analyzed by mass spectrometry.[2-6] In this study, we discuss optimization of methods to prepare, fractionate, and analyze intact ribosomal proteins.

Bottom-up studies commonly use pre-fractionation of complex samples to reduce sample complexity and improve the number of peptide identifications. Popular methods are strong cation exchange chromatography,[7] high-pH reverse phase chromatography,[8] and gel electrophoresis.[9] However, methods for pre-fractionation of intact proteins have been lacking. 2-dimensional gel electrophoresis is a powerful tool for resolving proteins, but recovering proteins intact from the

gel is problematic. Chromatographic methods such as ion exchange or size exclusion chromatography may also suffer from protein loss, possibly due to protein instability.[10] Recently, Gel Eluted Liquid FRaction Entrapment Electrophoresis (GELFREE) was developed specifically for pre-fractionation of complex protein samples.[11] The system separates intact proteins by SDS-PAGE, relying on the ability of SDS to stabilize proteins during separation. The main innovation of GELFREE is the ability to elute proteins "free of the gel," depositing the proteins into the liquid phase. This allows for far superior protein recovery than traditional SDS-PAGE, which requires that stained protein bands be cut from the gel. GELFREE fractionation has been used in top-down studies of yeast digest,[12] and mitochondria,[13] but ribosome studies have yet to take advantage of this technology.

While SDS is useful for stabilizing proteins, it causes signal suppression of proteins and peptides during electrospray ionization, and thus must be removed if mass spectrometric analysis will be performed. Three methods are commonly used to remove SDS from protein samples: proprietary detergent removal chromatography columns, methanol-chloroform-water extraction, and acetone precipitation. Chromatography columns for this purpose are manufactured by Thermo Scientific, and marketed as Pierce Detergent Removal Spin Columns. The resin used in these columns is composed of proprietary oligosaccharides, but is thought to use some sort of ion exchange mechanism. The columns are designed to bind detergents while allowing peptides and proteins to pass through.[14] Methanol-chloroform-water extraction involves adding methanol and chloroform in succession to the aqueous SDS solution. An organic chloroform layer forms at the bottom of the centrifuge tube while an aqueous methanol-water layer forms on top. SDS migrates to the aqueous layer, while ribosomal protein precipitates at the interface between the organic and aqueous layer. The SDS-containing aqueous layer is carefully removed to avoid

disturbing the precipitated protein, and additional methanol is added to the extraction. With the majority of the water removed, the chloroform and methanol form a single organic layer, transferring the precipitated protein to the bottom of the container. The protein pellet is then washed with fresh methanol and dried under vacuum.[15] Finally, acetone can be used to precipitate protein in a manner similar to methanol-chloroform-water extraction. Upon diluting the SDS-containing solution to 80% acetone, the solvents form a single layer and the protein gradually precipitates over the course of several hours. The protein can then be centrifuged into a pellet. This method requires more time than methanol-chloroform-water extraction, but requires less labor and is easier to reproduce.

Ribosomal proteins have been the subject of top-down proteomics studies in the literature.[16-19] However, these studies only measured the intact mass of the proteins, with no MS/MS data obtained. Some compensated for this by conducting top-down experiments in tandem with bottom-up experiments, using peptide analysis to identify PTMs and intact protein masses to confirm their existence on proteoforms. Furthermore, these studies also did not include pre-fractionation of their protein samples prior to LCMS analysis, limiting the number of proteoforms detected due to signal suppression of co-eluting proteins. In this work, we discuss methods to improve top-down analysis of ribosomal proteins through exploration of GELFREE fractionation for ribosomal proteins and fragmentation methods of intact protein ions.

MATERIALS AND METHODS

Yeast Growth

YIT613 FLAG-tagged ribosome yeast strain was obtained as a generous gift from Professor Toshifumi Inada at Nagoya University, Nagoya, Japan. YIT613 strain yeast was grown in YEPD media at 30°C, shaken at 200RPM. Cultures (250 mL) were grown in the presence of 0.0045 g tetracycline and 0.0045 g chloramphenicol, and then transferred into 2 L cultures. Log phase yeast was harvested at OD600 = 0.8, while stationary phase yeast was harvested after incubating for 48 hours. Yeast pellets were washed with lysis buffer (20 mM Tris-HCl, 2 mM magnesium acetate, 100 mM potassium acetate, 100 ug/mL cycloheximide), flash frozen with liquid nitrogen, and stored at -80°C.

Ribosome Preparation

Purification of ribosomes was adapted from published reports.[20, 21] One gram of wet yeast cells were resuspended in lysis buffer containing 10 uL/mL HALT protease inhibitor (Thermo Scientific) and 425-600 um acid-washed glass beads (Sigma) and lysed with a Biospec Products Mini-Beadbeater-1. Cell debris was pelleted at 13,000g for 5 minutes and the supernatant lysate was loaded onto a 2.5 mL column of ANTI-FLAG M2 Affinity Gel (Sigma). Bound ribosomes were washed with running buffer (50 mM Tris-HCl, 12 mM magnesium acetate, 100 mM ammonium chloride, 0.02% sodium azide, pH 7.4) and eluted with 100 ug/mL FLAG peptide. Ribosomes were partially concentrated by vacuum evaporation and pelleted by ultracentrifugation using a Type 70 Ti rotor at 50,000 rpm (max 257,000 g) for 3 hours. Concentrated ribosome samples were stored at -80°C. This procedure was found to yield ~100 ug of ribosomal protein by BCA assay.

Ribosomal Protein Release by RNAse

Ribosomal proteins were released from the ribosome by digesting the ribosomal RNA with commercial PureLink RNAse A (Invitrogen). Two ug RNAse was added to one batch of ribosomes and the enzymatic reaction was allowed to proceed for 45 minutes at room temperature.

Ribosomal Protein Release by Pressure Cycling

Suspended ribosomes were placed in a Pressure Biosciences Barocycler NEP 2320, after which the sample pressure was cycled between 35,000 psi and atmospheric pressure. 90 cycles of 50 seconds compression and 10 seconds at atmospheric pressure were applied. This process was also conducted with the addition of 20 ug RNAse to the sample prior to pressure cycling.

Ribosomal Protein Isolation by Acetic Acid

Ribosome proteins were isolated using the acetic acid method.[22] In short, ribosomes were treated with 66% Acetic Acid, 0.1M magnesium acetate for 2 hours, causing the solution to become cloudy. The solution was centrifuged at 17,000 g for 30 minutes, causing ribosomal RNA to pellet while ribosomal proteins remained in solution.

Ribosomal protein acetic acid solution was treated in one of three ways to remove or neutralize acetic acid. First, acetic acid solution was titrated with ammonium hydroxide until the pH reached 8. Second, acetic acid solution was transferred to a Harvard Apparatus 500 uL Fast Dialyzer chamber with a 500 Da Cellulose Acetate membrane and placed in 1 L of 25 mM

ammonium acetate at pH 8. Third, acetic acid solution was diluted with four volumes of acetone and stored at -20°C overnight, causing protein to precipitate. Precipitated protein was collected by centrifugation at 14,000 g for 10 minutes, after which the protein pellet was washed twice with acetone. A single "wash" involved removing supernatant acetone with a pipette, adding fresh acetone and repeating the centrifugation. Precipitated proteins were then allowed to gently dry in a chemical hood.

GELFREE Pre-fractionation

Following acetone precipitation, ribosomal proteins were resuspended either using GELFREE sample buffer containing 1% SDS and 0.05M DTT or 8M Urea with subsequent addition of GELFREE sample buffer and 0.05M DTT. 200 ug ribosomal proteins were fractionated on a "low mass" 12% cartridge, using the default recommended electrophoresis method. This involved a 2 hour separation, 70 minutes at 50 V and 50 minutes at 85V, during which 12 fractions were collected.

SDS Removal

GELFREE fractions were treated using one of three methods to remove SDS. The first was acetone precipitation, which was conducted identically to acetic acid removal as described previously. The second method was the use of 0.5 mL Pierce Detergent Removal Spin Columns. The spin columns were designed to retain detergent and allow proteins and peptides to pass through the column.[14]

The third method used methanol-chloroform-water extraction, where methanol and chloroform were added in series to the aqueous protein solution. Ribosomal protein precipitated at the interface between the resulting organic and aqueous layer. The top, SDS-containing aqueous layer was removed, and the protein pellet was washed with additional methanol and dried under vacuum.[15]

LCMS and Data Processing

Ten pmol of ribosomal proteins were injected onto a 15 cm, 100 um ID capillary column packed with Agilent PLRP-S 300A pore resin. A 3 hour 0.1% formic acid/acetonitrile gradient was used to elute proteins into a Thermo LTQ Orbitrap Velos. A data dependent top 2 algorithm was used to isolate proteins with either HCD or ETD fragmentation. HCD fragmentation was performed on peptides with 4+ charge or greater at a normalized collision energy of 30%, and ETD was performed on peptides with 4+ charge or greater with an activation time of 5-20 ms.

Protein spectra were manually assessed and MS/MS spectra showing good protein fragmentation were Xtracted using Xcalibur Qualbrowser. Individual Xtracted spectra were imported into Prosight PC and searched against a Uniprot database containing only *Saccharomyces cerevisiae* ribosomal proteins. Proteins were checked for initial methionine truncation and n-terminal acetylation.

RESULTS AND DISCUSSION

Ribosomal proteins were produced using the methods described in Chapters 2 and 3. While these methods were adapted from existing reports,[20, 21] some changes were made to optimize the samples for analysis by mass spectrometry. Early yeast cultures showed signs of contamination, which was confirmed by optical microscopic imaging (Fig 4.1). Although ANTI-FLAG affinity chromatography of ribosomes could be expected to capture principally yeast ribosomal proteins, trace amounts of non-specifically bound bacterial proteins would be enough to increase sample complexity in a mass spectrometry analysis. Attempts to sterilize work surfaces and carefully monitor ongoing cultures alleviated the problem somewhat, but failed to eliminate it. Finally, the antibiotics tetracycline and chloramphenicol were used to eliminate all possible bacterial contamination in the small (250 mL) cultures prior to transfer into the large (2 L) cultures. This strategy kept the yeast cultures clear of bacterial contamination while limiting the quantity of antibiotics expended.

Existing ANTI-FLAG chromatography methods involved buffers that were not optimal for our purposes. ANTI-FLAG Affinity gel requires salt to remain stable, encouraging the use of potassium chloride in the running buffer. However, trace amount of potassium is well-known by the mass spectrometry community to cause signal suppression of proteins and peptides. Thus, we replaced potassium salts with ammonium salts; ammonium is a volatile ion, making it far more compatible with mass spectrometry. Another problematic buffer component was DTT, ostensibly used to prevent non-specific protein binding to the ANTI-FLAG column. However, DTT decreases lifetime of the affinity gel, and thus would require costly replacements of the affinity gel. Removing DTT from all buffers allowed for individual ANTI-FLAG columns to purify more than ten samples before requiring replacement, an improvement from the commercially-

advertised three samples per column. Glycerol was briefly considered as a buffer component for its ability to stabilize purified protein complexes.[23] However, glycerol caused problems when attempting to concentrate ribosome samples after purification, and glycerol was omitted without apparent cost to protein stability.

The ribosome is a remarkably stable complex of RNA and protein molecules, prompting consideration of several different methods for isolating ribosomal proteins from the ribosome complex. The effectiveness of these methods were tested by SDS-PAGE separation of the ribosomal components, as shown in Fig 4.2 and 4.3. First, ribosomes were boiled extensively in SDS-PAGE buffer for 5 to 15 minutes, but this was largely ineffective, with most of the protein staying at the top of the SDS-PAGE gel in the form of intact ribosomes. Next, ribosomes were treated with RNAse I, with the intention of lysing exposed sections of the rRNA. This proved fairly effective at separating the ribosomal components, with gel bands appearing from 5 to 50 kDa. However, the band pattern is different from that expected of completely isolated ribosomal proteins,[21] suggesting that small lengths of cleaved RNA remained bound to ribosomal proteins, allowing for complexes of two or more bound proteins to persist.

Pressure cycling using a Pressure Biosciences Barocycler NEP 2320 was tested for separating ribosomal components. Pressure cycling has been used to lyse tissue and enhance enzymatic digestion.[24] It was hypothesized to be able to dismantle the ribosome or, barring that, "loosen" components of the ribosome to allow better access for RNAse to cleave ribosomal RNA. However, SDS-PAGE analysis shows that pressure cycled samples were indistinguishable from ribosomes that were intact prior to the addition of SDS-PAGE sample buffer, and samples pressure cycled in the presence of RNAse were indistinguishable from samples that were treated

with RNAse at atmospheric pressure. Thus, the barocycler was ruled inadequate for separating ribosomal components.

Finally, ribosomes were treated with 66% acetic acid and 0.1M magnesium acetate. This has been shown to cause ribosomal RNA to precipitate, while the ribosomal proteins remain in solution due to their highly charged nature.[22] Acetic acid is very compatible with mass spectrometry, and a simple dilution with water to 10% acetic acid caused no problems for the UPLC autosampler. Thus, 66% Acetic Acid treatment proved most effective for isolating ribosomal proteins for mass spectrometry.

Ribosomal proteins in 10% acetic acid were fractionated using a 15 cm C18 reverse phase capillary column and analyzed using a LTQ Orbitrap Velos (Thermo). Capillary columns packed with polymer-based PLRP-S resin and silica-based C18 resin were both used, but no obvious difference in performance was observed. A data dependent top 2 algorithm was used to isolate proteins with 4+ charge or greater and conduct HCD or ETD fragmentation. Ionized proteins were fragmented with HCD and ETD fragmentation, both of which achieved good fragmentation spectra. ETD fragmentation of ribosomal proteins was tested using activation times ranging from 5 to 20 ms, but no significant correlation between activation time and fragmentation efficiency was found (Fig 4.4).

Protein spectra were manually assessed using Xcalibur Qualbrowser to find likely candidates of fragmented proteins. Co-elution of two or more proteins was commonly observed, with smaller, more readily-ionized proteins greatly suppressing signal for other co-eluting proteins. MS/MS scans involving intact masses greater than 6 kDa, ions of high charge, and multiple fragmentation peaks were manually Xtracted into separate data files using Xcalibur

Qualbrowser. Individual Xtracted spectra were imported into Prosight PC and searched against a Uniprot database containing only *Saccharomyces cerevisiae* ribosomal proteins. Due to time constraints, only a subset of high quality MS/MS spectra were analyzed. Among 7 minutes' worth of data collected between the 60 minute and 85 minute mark of the chromatographic separation, 64 high quality MS/MS spectra were Xtracted, and 12 ribosomal proteins were identified with high confidence, with two proteoforms detected for ribosomal protein S28B (RPS28B) (Table 4.1). Although Prosight PC includes a "high-throughput" feature that automatically Xtracts and searches all MS/MS spectra from a single LCMS .raw file, this feature was unable to assess the top-down ribosomal protein data. Extended collaborative efforts with Thermo Scientific technicians were unable to resolve this issue. Thus, although software analysis experienced unresolved problems, ribosomal proteins were successfully detected, fragmented, and identified by top-down mass spectrometry.

In order to reduce co-elution of ribosomal proteins, a method of pre-fractionation was needed to reduce sample complexity. For this purpose, GELFREE fractionation was chosen due to its ability to fractionate and stabilize proteins for top-down proteomics analysis.[10, 11] However, acetic acid changed the sample buffer pH and disturbed the ion concentration of the sample buffer, causing the stacking step of the GELFREE separation to fail. To remove acetic acid, dialysis in ammonium acetate buffer (pH 8) was attempted using a 0.5 mL dialysis chamber. However, protein concentration was too low for this approach, and negligible amounts of protein were recovered after dialysis. Titration with ammonium hydroxide to pH 8 matched the GELFREE sample buffer pH, but the resulting ammonium acetate salt interfered with the GELFREE separation, causing similar problems as the original acetic acid.

Finally, the protein was precipitated in 80% acetone at -20°C overnight. The precipitated protein was collected by centrifugation, and the protein pellet was washed with additional acetone to remove residual acetic acid. This proved very effective at removing acetic acid, and has been used in the literature.[25] However, the following step usually involves resuspending the pellet in urea or dilute formic acid. First, urea was used to redissolve the protein pellet, which appeared to dissolve the protein very quickly. Unfortunately, urea was found to alter the electrophoretic elution profile of the GELFREE separation, making it incompatible. Next, the protein pellet was dissolved in 1% acetic acid and titrated with ammonium hydroxide to pH 8. Although it was previously demonstrated that titration of 66% acetic acid was not an option for GELFREE samples, titration of 1% acetic acid proved to create little enough salt that the GELFREE separation could still sometimes run effectively. However, even this low amount of salt made the method unreliable, with some samples resolving well and others resolving poorly. Finally, boiling the ribosomal protein pellet in GELFREE sample buffer successfully redissolved some of the protein, albeit with poor yield. Still, this method was compatible with GELFREE separation, and enough protein was redissolved to obtain a separation profile of the ribosomal proteins (Fig 4.5).

Following every GELFREE fractionation, SDS must be removed from the protein fractions to allow analysis by mass spectrometry. As an ionic detergent, SDS is known by the mass spectrometry community to cause signal suppression of peptides and proteins, similar to sodium or potassium salts. Three methods were tested to remove SDS from ribosomal samples: Pierce Detergent Removal Spin Columns, methanol-chloroform-water extraction, and acetone precipitation. Protein recovery for each method was tested by running recovered proteins on a silver-stained SDS-PAGE gel (Fig 4.6). Protein recovery for every method was very poor, with

methanol-chloroform-water proving to be slightly more effective than its counterparts. For detergent removal chromatography, it is hypothesized that the ion exchange mechanism binds highly charged ribosomal proteins to a higher degree than lower charged proteins such as bovine serum albumin (BSA), causing low protein recovery.

For methanol-chloroform-water extraction and acetone precipitation, however, low protein recovery appears to be due to an inability to resolubilize ribosomal proteins after precipitation. Acetone precipitation was observed to produce a small, visible pellet, estimated on the order of 1 ug of protein. To test if the proteins recovered by acetone precipitation were indeed yeast ribosomal proteins, the protein pellets were dissolved in urea, digested with trypsin, and analyzed by mass spectrometry as discussed in Chapter 2. The majority of yeast peptides identified were ribosomal peptides, establishing that the protein pellets were indeed ribosomal proteins. Acetone precipitation was also conducted with the use of sodium chloride and ammonium acetate salts, which has been suggested to improve protein yield. However, no difference was observed for precipitated ribosomal proteins. 10% formic acid was used to resolubilize precipitated proteins from both acetone precipitation and methanol-chloroform-water extraction, causing the protein pellets to apparently dissolve. However, LCMS analysis of the formic acid solution detected no proteins, suggesting that the protein pellet was suspended as one aggregate mass. From these observations, it was concluded that ribosomal proteins were successfully precipitated by methanol-chloroform water and acetone treatment, but could not be resuspended in SDS-PAGE buffer or formic acid. The forementioned urea suspension and tryptic digest experiment suggests that urea may be able to resuspend ribosomal proteins, but the urea must be subsequently removed with a desalting column to make the sample compatible with mass spectrometry. Such a desalting step was attempted using a variety of off-line desalting

columns, but no protein was detected on a silver stained SDS-PAGE gel or BCA analysis. Thus, none of the SDS removal methods tested were able to recover ribosomal proteins in a manner compatible with mass spectrometry.

CONCLUSIONS

In this work, we discussed the optimization of methods to isolate, fractionate, and analyze intact yeast ribosomal proteins. While we did successfully identify ribosomal proteins by top-down mass spectrometry, identifications were hindered by inadequate software. The top-down proteomics field is still in its infancy, and difficulties like this are to be expected. However, we were able to make enough progress through manual data analysis to realize that without pre-fractionation, top-down ribosomal protein analysis would be limited by signal suppression of co-eluting proteins. Thus, we decided to focus our efforts on pre-fractionation, with the intention of resolving the software issue once we achieved chromatographic resolution of ribosomal proteins. Unfortunately, we came to the conclusion that although GELFREE successfully fractionates ribosomal proteins, it is not possible to remove the SDS with existing methods while keeping the ribosomal proteins solubilized in a buffer compatible with mass spectrometry. SDS removal by the methods we tested has seen success for many other samples,[12, 13, 26] but we believe that this problem stems from the unusual highly charged nature of ribosomal proteins, making them very difficult to redissolve following denaturation and precipitation in the presence of SDS. It is notable that purified histone proteins, which are highly charged to a similar degree, have also not been successfully fractionated by GELFREE in the literature despite their frequent study by top-down mass spectrometry. We hope that this work will lead towards successful pre-fractionation

of ribosomal proteins in the future, allowing chromatographic resolution and top-down quantification of ribosomal proteins to become a reality.

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Table 4.1

Protein	Modification	Mass	Frag. Ions	E-value	Rxn Time (	M/Z	Charge
RPL24A		17602.9	25	3.14E-08	12	801.61	22
RPL24B		17536.9	13	3.39E-22	12	702.77	25
RPL26A	-Met	14094	24	1.04E-44	6	642.01	22
RPL30	-Met	11277.2	33	1.31E-24	12	1028.4	11
RPL31A	-Met	12814.1	30	6.45E-22	12	916.67	14
RPL34B	-Met	13501.5	31	2.00E-56	6	644.13	21
RPS13	-Met	16887.3	34	2.84E-10	12	890.19	19
RPS24A		15319.4	8	2.58E-12	12	693.71	22
RPS26A	-Met	13365.3	22	2.96E-44	12	704.61	19
RPS28A		7587.18	8	3.29E-03	6	764.34	10
RPS28B	-Met	7429.13	27	2.02E-22	12	695.03	11
RPS28B		7560.17	14	9.61E-16	6	846.04	9
RPS29A	-Met	6525.26	14	1.64E-13	6	726.04	9

Table 4.1. Ribosomal proteins identified by top-down mass spectrometry. MS/MS spectra showing good fragmentation of proteins were manually Xtracted using Qualbrowser and imported into Prosight PC. Spectra were searched against a Uniprot database containing *Saccharomyces cerevisiae* ribosomal proteins. E-value = N x p(n), where E-value is the expectation value, N is the number of protein sequences in the database, and p(n) is the probability that the detected fragment masses match the protein's theoretical fragment masses by chance. A lower E-value indicates a stronger match. Initial mass is only considered when two or more possible proteins have matching E-values.

Figure 4.1

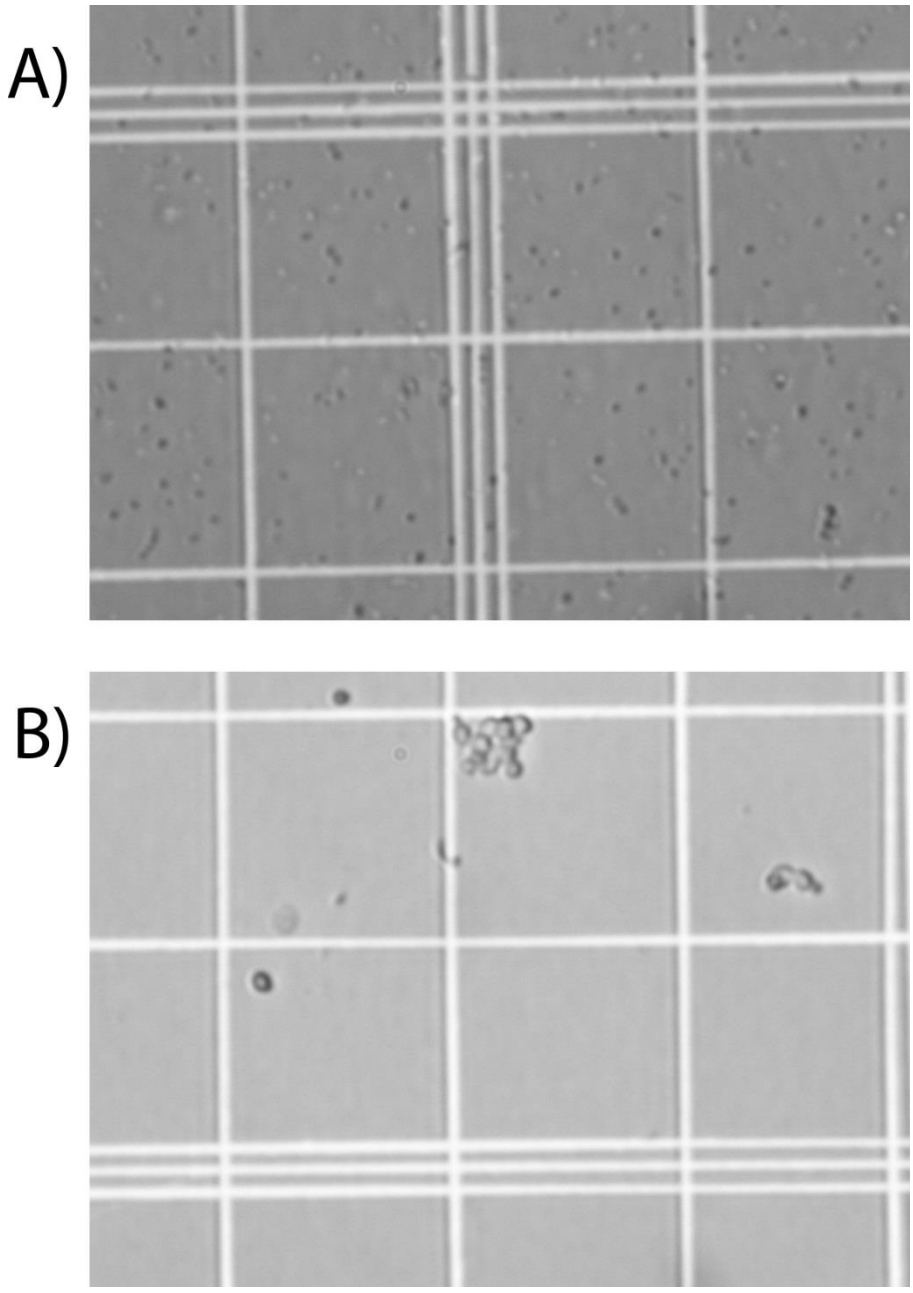

Figure 4.1. Microscope images of yeast cultures. A. Yeast contaminated with unidentified bacteria. B. Yeast cells grown in the presence of antibiotics tetracycline and chloramphenicol. Antibiotics keep the culture clear of bacterial contamination.

Figure 4.2

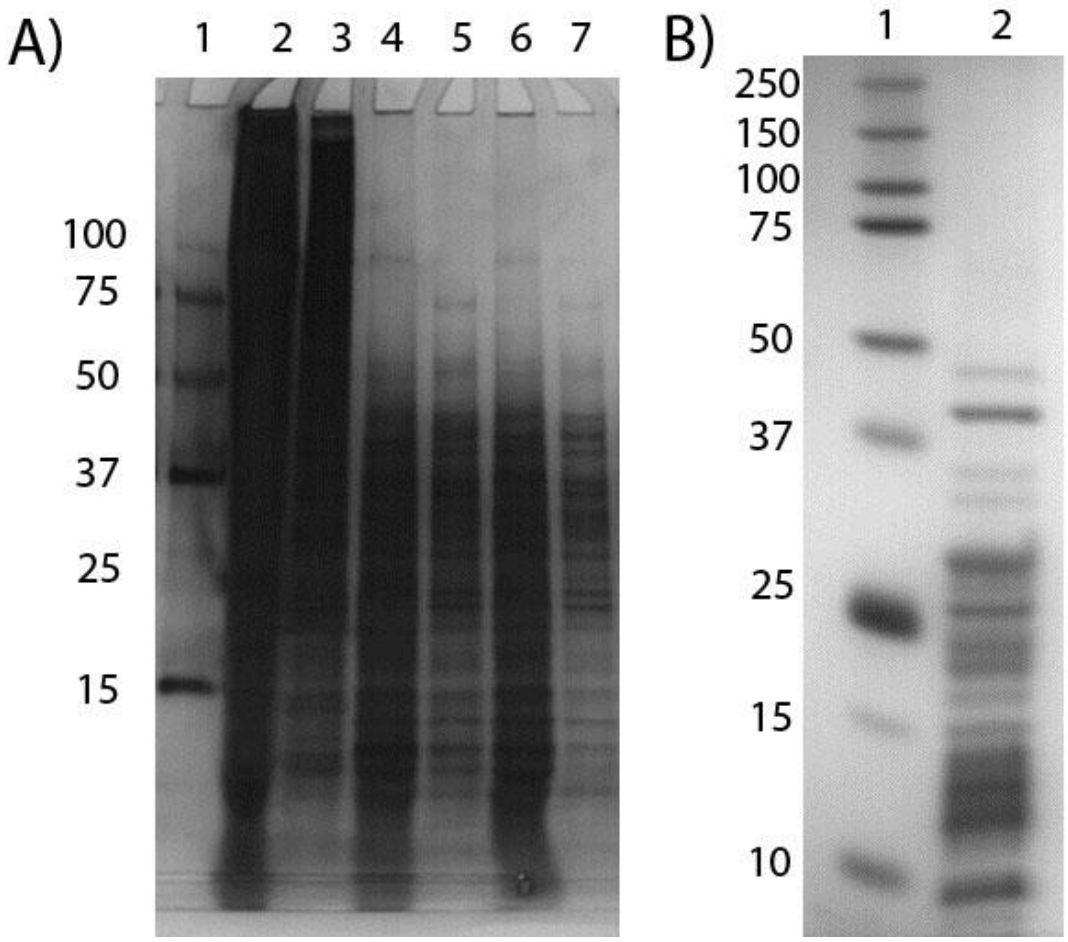

Figure 4.2. Silver stained SDS-PAGE 5-20% gels of ribosomes showing different protein isolation methods. Gel A, Lane 1: Molecular weight markers. Lanes 2 and 3: Ribosomes boiled in SDS-PAGE buffer for 5 minutes. Lanes 4 and 5: Ribosomes lysed with RNAse at room temperature. Lanes 6 and 7: Ribosomes lysed with RNAse and treated with pressure cycling. Gel B, Lane 1: Molecular weight markers. Lane 2: Ribosomes treated with acetic acid, precipitated in acetone, and resuspended in SDS-PAGE buffer.

Figure 4.3

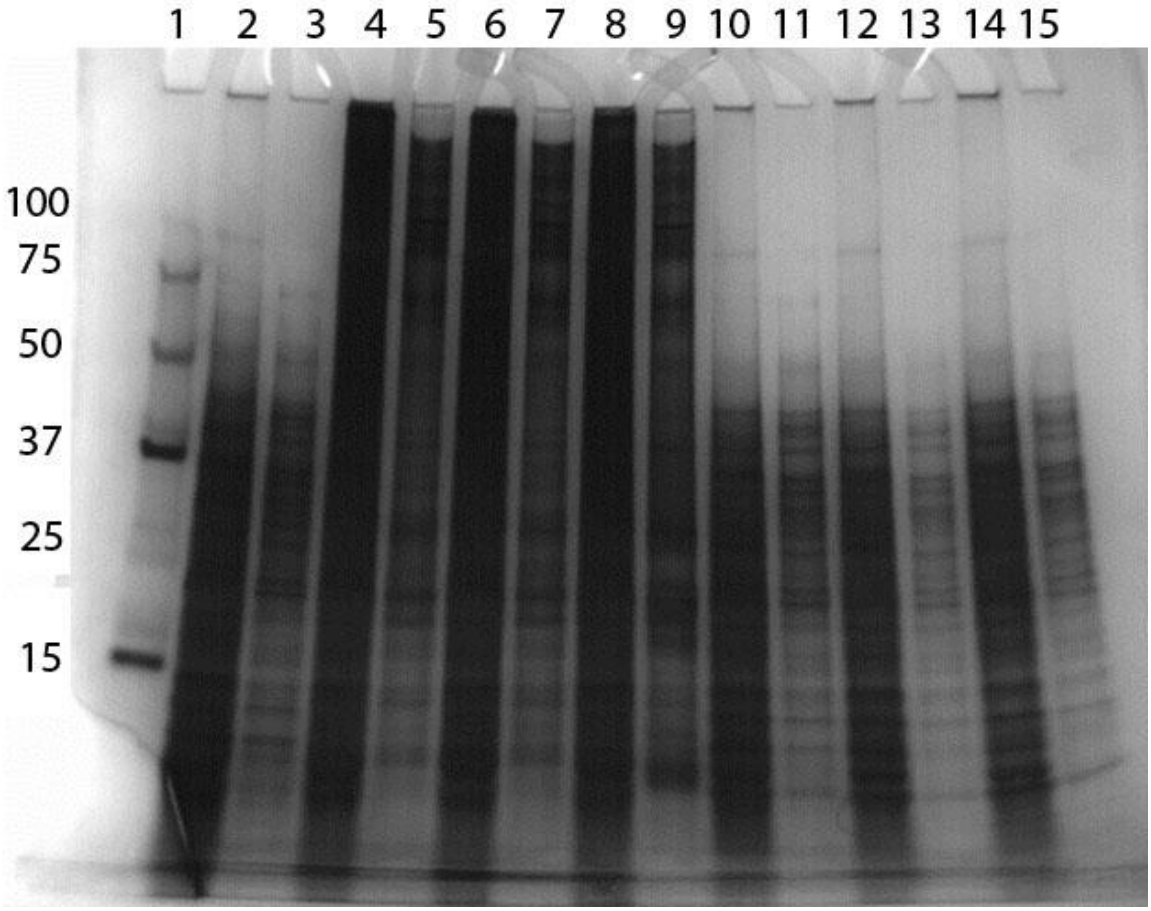

Figure 4.3. Silver stained SDS-PAGE 5-20% gel of ribosomes showing different protein isolation methods. Lane 1: Molecular weight markers. Lanes 2 and 3: Ribosomes lysed with RNAse at room temperature. Lanes 4-9: Ribosomes treated with pressure cycling in the absence of RNAse. Lanes 10-15: Ribosomes lysed with RNAse and treated with pressure cycling.

Figure 4.4

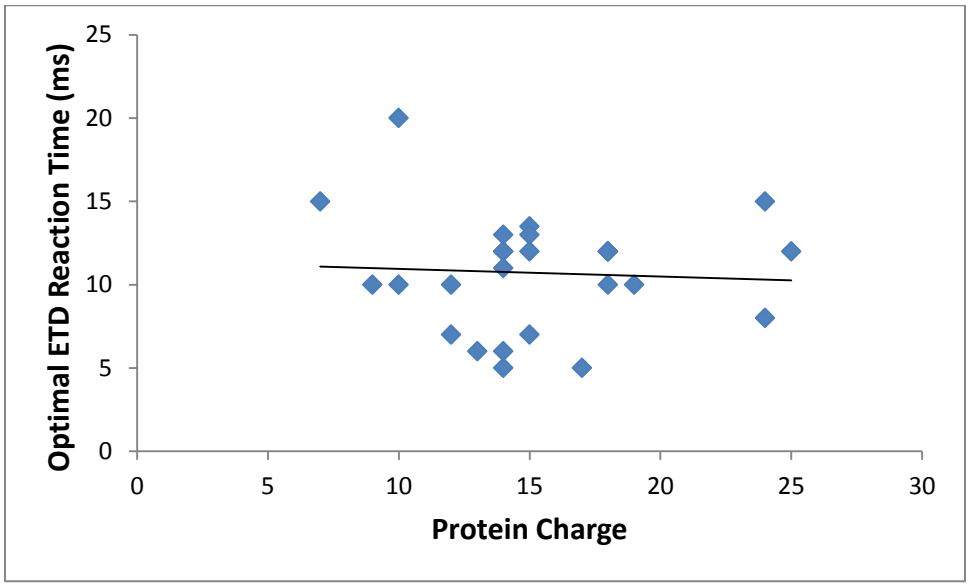

Figure 4.4. Plot of protein charge versus optimal ETD reaction time. Optimal reaction time was assessed manually by comparing ETD fragmentation spectra after 5, 10, 15, and 20 ms. Optimal reaction times were assessed on a weighted scale. I.e. if 10 ms yielded the best fragmentation, 10 was assigned as the optimal reaction time. If 10 ms and 15 ms both yielded equally good fragmentation, 7.5 was assigned as the optimal reaction time. A linear regression for the data was calculated, and R^2 value was found to be 0.0035, indicating no linear correlation between protein charge and optimal ETD reaction time.

Figure 4.5

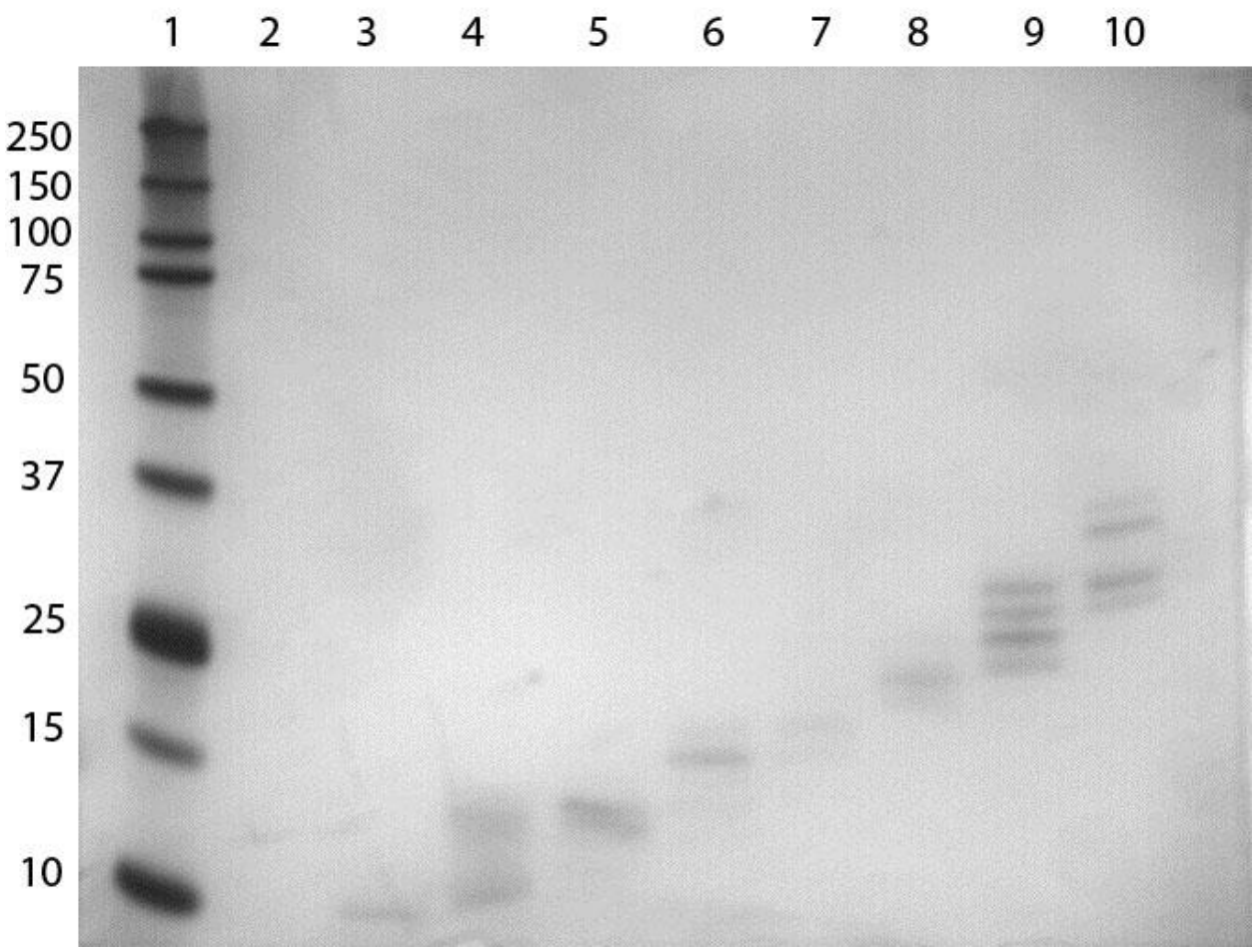

Figure 4.5. Silver stained SDS-PAGE Bis-Tris 12% gel of ribosomes showing different protein isolation methods. Lane 1: Molecular weight markers. Lanes 2 and 3: Ribosomes boiled in SDS-PAGE buffer for 5 minutes. Lanes 4 and 5: Ribosomes lysed with RNAse at room temperature. Lanes 6 and 7: Ribosomes lysed with RNAse and treated with pressure cycling.

Figure 4.6

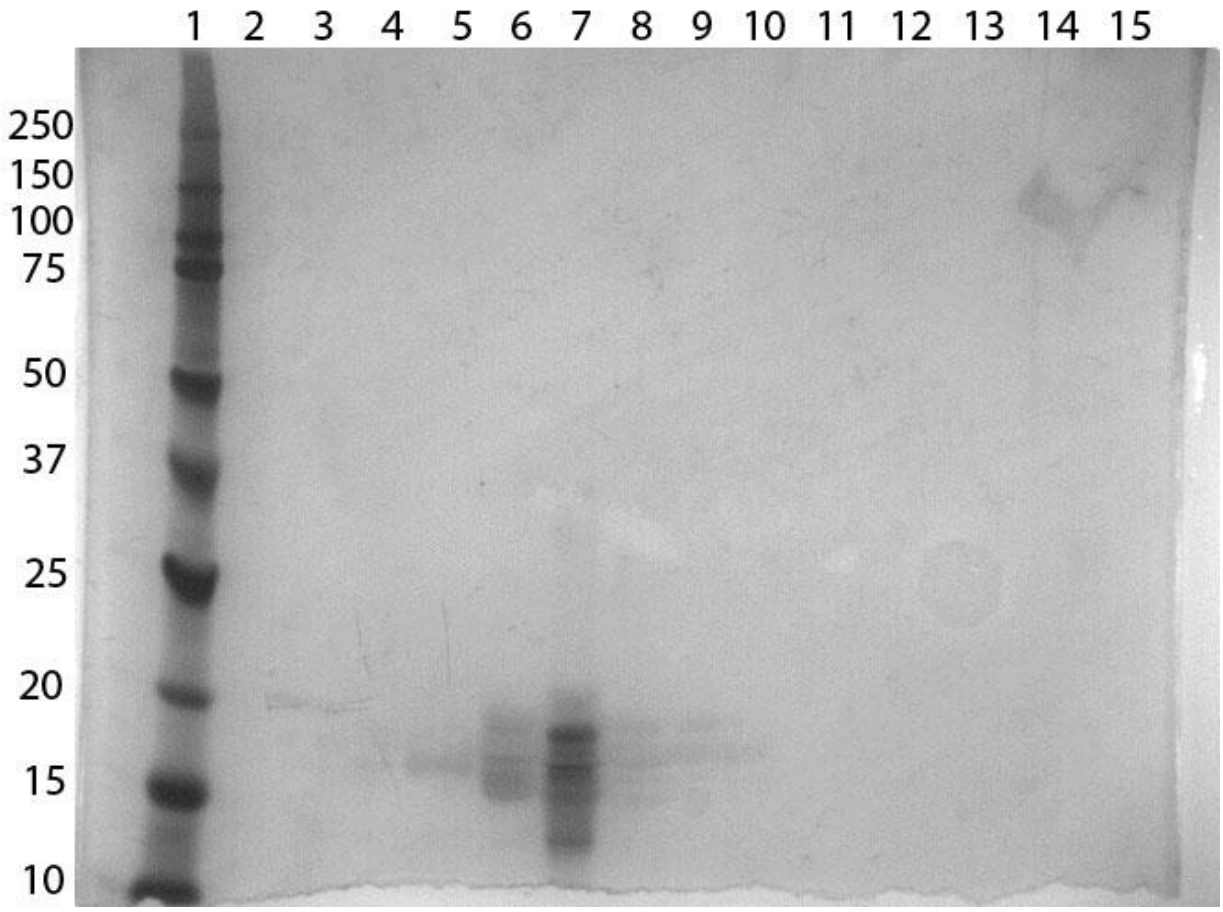

Figure 4.6. Silver stained SDS-PAGE 5-20% gel of ribosomes showing different protein isolation methods. Lane 1: Molecular weight markers. Lanes 2-3: GELFREE Fraction 6 (GF6), Acetone. Lanes 4-5: GF7, Acetone. Lanes 6-7: GF8, Methanol-Chloroform-Water. Lanes 8-9: GF9, Acetone with 1% TFA. Lanes 10-11: GF10, Strong Cation Exchange Column. Lanes 12-13: GF11, Acetone. Lanes 14-15: GF12, Pierce Detergent Removal Spin Column. Protein bands are visible for acetone and methanol-chloroform-water treated samples.

CHAPTER 5:

LED-INDUCED FLUORESCENCE QUANTIFICATION OF PEPTIDES

While previous chapters focused on quantification of peptides and proteins by mass spectrometric peak intensity, we have also explored intrinsic fluorescence an alternative method of quantification. Liquid chromatography coupled with mass spectrometry (LC/MS) has proven to be invaluable in identifying proteins from a complex mixture, but quantitative analysis is inherently hindered by differing ionization efficiencies. To solve this problem, we have developed an approach for absolute protein and peptide quantification that integrates an LED-induced fluorescence system into a conventional LC/MS format. This method allows peptides and proteins to be quantified by native fluorescence and identified by conventional shotgun mass spectrometry techniques. Thus, as fluorescence data is taken prior to ionization at the electrospray tip, quantitative data can be acquired without the problem of differing ionization efficiencies. Here, we discuss optimization of the fluorescence system, and present data obtained from analysis of proteasomal proteins.

INTRODUCTION

Mass spectrometry has become the method of choice for analysis of complex biological samples, a trend made possible by the soft ionization methods of electrospray ionization (ESI) and matrix-assisted laser desorption/ionization (MALDI) which were recognized by the 2002 Nobel Prize in chemistry. The majority of LC/MS proteomics experiments today consist of the enzymatic digestion of a protein sample, separation of the resulting peptides by liquid

chromatography, electrospray ionization of peptides into the gas phase, analysis and fragmentation of the protonated peptides inside the mass spectrometer, and finally mass analysis of the resulting fragment ions. The resulting MS/MS spectra are then compared to one or more protein sequence databases for identification.[1]

The nature of this analysis was originally only qualitative, as differing charge state, peptide length, amino acid composition, or posttranslational modifications result in differing ionization efficiencies for the peptides analyzed.[2] Co-elution of two or more peptides can also cause problems due to signal suppression, as the competition of ions for access to the droplet surface and/or charge causes reduced ion intensity for peptides that are less-readily ionized.[3] To circumvent these obstacles, two general strategies for high-throughput quantitative mass spectrometry-based proteomics are widely used: label and label-free analysis. Each strategy is subdivided into various techniques aimed at providing relative quantification of protein abundances inferred from peptides identified and quantified from enzymatically-digested protein mixtures.

Isotopic labeling techniques involve introducing heavy-isotopes at various points in the sample preparation so that corresponding "heavy" and "light" peptides will be detected in the same spectra for quantitative comparison. In 2002, Mann published the method of stable isotope labeling by amino acids in cell culture (SILAC) in which two cell growths are conducted, one with the addition of "heavy" ^{13}C6-arginine and ^{13}C6-lysine to the growth medium. The two samples are combined and then subjected to purification, digestion, and analysis, ensuring that all variables during this process are consistent for both the "heavy" and "light" proteins.[4] While this method may be the most accurate quantification method, this process is time-intensive and limited to comparison of two samples. ITRAQ involves the tagging of up to eight samples with

mass-calibrated tags that then detach during fragmentation inside the mass spectrometer. The "reporter ions" then allow for quantification. Using stable isotope tagging and isotope dilution require the use of expensive isotopic reagents and, in the case of tagging, may introduce bias due to labeling chemistry. Heavy labeled tagging methods such as isotope-coded affinity tag (ICAT) and isotope tags for relative and absolute quantification (iTRAQ) allow for quantitative comparison of up to eight samples. ICAT modifies cysteine residues with tag molecules labeled with zero or eight deuterium atoms.[5] This reduces sample complexity due to the rarity of cysteine in most proteomes, but still only allows for the comparison of two samples. iTRAQ is a more recent labeling technique that tags the n-termini and lysine residues with isobaric tags that detach upon fragmentation by the mass spectrometer. As the isobaric tags are of a lower m/z than most peptides, up to eight samples may be compared at once without overlap of the isotopic envelopes. However, these labeling techniques may suffer from quantitative bias due to varying efficiencies of the tagging reactions. A method known as absolute quantification of proteins (AQUA) involves the use of labeled synthetic peptides as internal standards for specific peptides of interest. While AQUA peptides can be added directly to the sample to be analyzed and involve no labeling bias, the selection of peptides to be used as internal standards requires prior knowledge of the sample to select and synthesize suitable standard peptides.[6] Cost is an issue for all isotopic labeling techniques.

Two label-free quantification techniques are common today: signal intensity and spectral counting. Signal intensity involves finding the peak intensities of the extracted ion chromatogram, which is constructed from the measured MS peaks' intensity over time. This intensity is then compared for identical peptides across different experiments.[7] This method requires frequent sampling of the MS peak to acquire accurate peak shapes, which limits the

number of MS/MS scans that can be taken. Thus, quantitative data is taken at the expense of peptide identification. Spectral counting judges protein quantity by counting the number of MS/MS scans that are attributed to that protein. Thus, frequent MS/MS data is advantageous for quantification, which similarly benefits peptide identification. However, spectral counting has its critics in that it does not directly measure any physical property of the peptides analyzed. Both label-free methods are susceptible to experimental conditions as different temperature, separation conditions, retention time and sample handling can affect peak shape. Thus, while label-free methods invoke no added cost or sample preparation, they are unreliable for comparing nonconsecutive experiments.[8]

All of the aforementioned quantification techniques are limited to relative quantification. Labeling techniques are costly and require time and effort to prepare samples for quantitative study. Label-free techniques require attention to keep experimental conditions consistent, and can be lacking in accuracy. Thus, there is a clear need for a convenient, low-cost method for absolute quantification of peptides and proteins.

Native fluorescence satisfies these requirements. Conventional proteomic samples can be analyzed without additional preparation or expensive reagents. Retention time and peak shape do not affect the measured fluorescence peak area. Most importantly, fluorescence allows absolute quantification, which can be used to calculate biologically relevant information such as protein copy number per cell.

Native fluorescence originates from tryptophan, tyrosine, and phenylalanine residues. Of these three, tyrosine has the largest extinction coefficient, the highest quantum yield, and the longest emission wavelength. Thus, not only does Tryptophan produce the most fluorescence, it

also can be measured at a wavelength where emissions from tyrosine and phenylalanine are minor (Lakowitz 2006). Laser-induced fluorescence has been used as a detection method for protein capillary electrophoresis separations for years, using the 275.4 nm band of an argon-ion laser.[9] In 2005, Sluszny et al. published a LED-induced fluorescence system as a more economical approach.[10] We have adapted this system for our capillary LC detection methods.

Here, we discuss work done to optimize our LED-induced fluorescence quantification system. The inner and outer diameters of the capillary were optimized to improve signal and reduce background. Fluorescent standards were detected in different running buffers to test the effect of pH on fluorescence signal. A cold air gun was used to test the effect of LED temperature on fluorescence signal. Finally, a plastic shield was constructed to more efficiently block out ambient light from the photomultiplier tube (PMT).

MATERIALS AND METHODS

LED-induced Fluorescence

Components of the LED-induced fluorescence system are shown in Figure 5.1. A 200 um ID capillary HPLC column with integrated spray tip was mounted to a breadboard by sliding it through a length of PEEK tubing sleeve which was then secured into a locating groove on a fixed metal rod. The last 1.4 cm of capillary containing the detection window protruded over a 280 nm UV-LED (UVTOP-280-TO39BL, Sensor Electronic Technology, Columbia, SC.) The LED was powered by a constant-current power source (LED Power Supply Plus, Sandhouse Design, Dunedin, FL) set up to 30 mA for a maximum output power of 400 µW. The capillary was positioned 11 cm above the center of the LED's integrated ball lens. The LED was placed inside

an aluminum sleeve which supported a bandpass interference filter with a center wavelength of 280 nm and a bandwidth of 20 nm (Semrock, Rochester, NY). A 4 mm diameter silica ball lens (ISP Optics, Irvington, NY) was mounted on a 3-D translation stage and positioned above the center of the LED, immediately below the capillary. Fluorescence was collected from the capillary at a right angle to the excitation light with a fused silica plano-convex lens with a diameter of 0.5 inch and a focal length of 19 m (Newport, Irvine, CA) mounted on a 3-D translation stage. The fluorescence was directed through a 0.5 inch diameter aperture positioned 95 mm beyond the collection lens. This aperture was at the entrance of a black delrin tube (1 inch id, 75 mm long) that housed a long pass colored glass filter with a cut-on wavelength of 324 nm (Newport) followed by a bandpass interference filter with a center wavelength of 357 nm and a bandwidth of 44 nm (Semrock). The tube was mounted on the entrance to a R928 photomultiplier tube (Hamamatsu Photonics, Hamamatsu, Japan). The entire system was mounted on a 3-D translation stage used to adjust the positioning of the spray tip in front of the mass spectrometer inlet without disturbing the optical alignment.

The signal from the PMT was passed through a home-built current-to-voltage converter. The voltage was then sampled using a PCI-6035E card (National Instruments, Austin, TX) within a personal computer running Windows XP. Signal processing was done using a program written in LabVIEW 8.5 (National Instruments). Typically, the signal was sampled at a rate of 100 kHz and 104 samples were averaged to produce one recorded data point resulting in the generation of ten data points per second.

LCMS methods were used as described in Chapter 2.

Cold Air Gun

The LED was cooled using an external air gun (model 60071, Airtx.) Compressed air from a 250 cubic foot tank was run through the compressed air gun at 80 psi and focused on the UV-LED for 5 minutes. Compressed air was exhausted at 283 L/min.

RESULTS AND DISCUSSION

The inner and outer diameters of the capillary were optimized to improve signal and reduce background. Figure 5.2 shows fluorescence signal of 10 uM tryptophan in capillaries of 360 um and 200 um outer diameter (OD). Although background was found to be identical for the two capillaries, the 200 um OD capillary produced marginally better signal, most likely due to less refraction of fluorescent light produced by the tryptophan analyte. However, this improvement came at the cost of capillary stability, so 360 um was chosen as the more reliable OD. Inner diameter was also tested, as shown by Figure 5.3. The largest ID, 200 um, produced the most intense fluorescence signal, as predicted. The larger ID capillary possessed a larger cross-section, allowing more tryptophan molecules to be excited by the LED at once. Thus, 360 um OD and 200 um ID was chosen as the optimal dimensions for the capillary.

Another approach to improving fluorescence signal was to increase the LED output intensity, but operating the LED at a higher current was found to reduce LED lifetime. To enhance LED intensity without increasing the supplied current, we examined the effect of cooling the LED temperature on the intensity of the light produced. A cold air gun was used to cool the LED by 5°C, upon which UV-LED intensity increased by 7%, as measured by the photomultiplier tube (Fig 5.4). This increase matched reported intensity gains where cooling the LED to 3°C led to a 1.6-fold increase in output light.[10] However, to achieve this rate of cooling,

compressed air was expelled at 283 L/min, a rate that would exhaust the tank in 30 minutes. Thus, we concluded that cooling by compressed air was impractical for regular fluorescence measurements. A peltier device was considered for LED cooling, but locating a heat sink outside of the fluorescence system container proved problematic. Thus, LED-induced fluorescence operation remained at room temperature.

Finally, the system housing was optimized to reduce both fluorescence and mass spectrometric background. Originally, a covering of black cloth was used to shield the PMT from ambient light. However, the presence of the cloth created noticeable background peaks in the mass spectrum. Thus, a plastic shield was constructed to replace the cloth. Not only did the plastic housing eliminate background peaks from the mass spectrum, it also blocked ambient light more efficiently. Thus, the fluorescence system was operated with the plastic shield from that point onward.

These optimizations proved useful when the fluorescence system was used by Russell et al. to analyze peptides and proteins,[11] including intact proteins of the *Arabidopsis thaliana* 26S proteasome.[12] Thirteen tryptophan-containing proteins of the 26S proteasome were detected, as is shown as an example in Figure 5.5. Quantification of intact proteins by LED-induced fluorescence was found to have a linear response to protein concentration, and significantly more precise than mass spectrometric peak intensity (Figure 5.6).

CONCLUSIONS

In this work, we discussed the optimization of an LED-induced fluorescence system for quantifying peptides and proteins in tandem with mass spectrometric analysis. While cooling the

UV-LED was found to be impractical, optimizing the column dimensions and constructing an improved housing for the system improved induced fluorescence signal and reduced background. This inexpensive, modular system was used to effectively quantify peptides and proteins, and it can be readily adapted to measure any emission and excitation wavelengths by exchanging the UV-LED and optical filters, allowing for numerous possible alternative applications.

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Figure 5.1

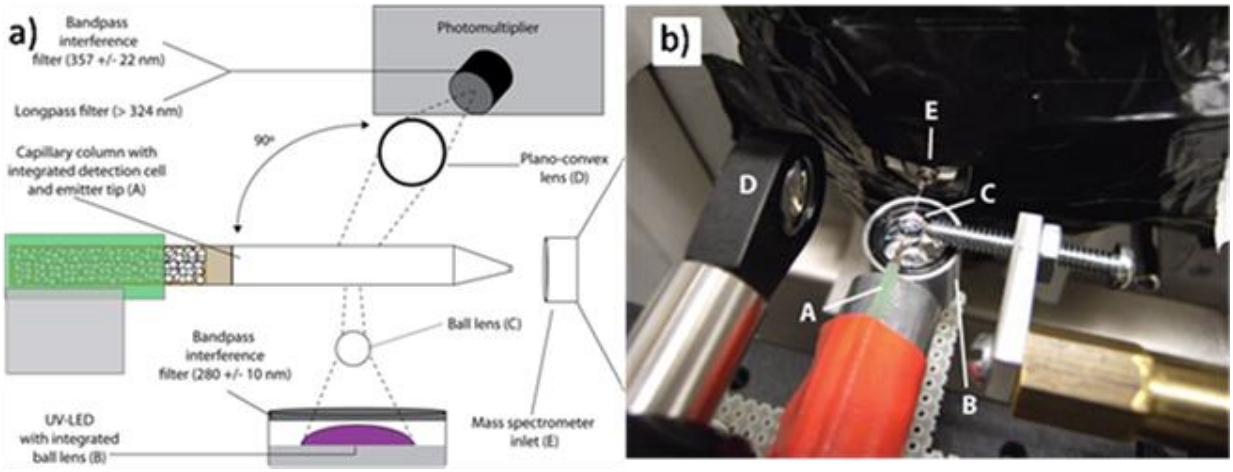

Figure 5.1. a) Graphical representation and b) actual image of the fluorescence excitation and detection system interfaced to a mass spectrometer. A = Capillary column (200 µm) with integrated detection cell and emitter tip, B = UV-LED with integrated ball lens, C = focusing ball lens, D = fluorescence detection lens (plano-convex), E = mass spectrometer inlet.

Figure 5.2

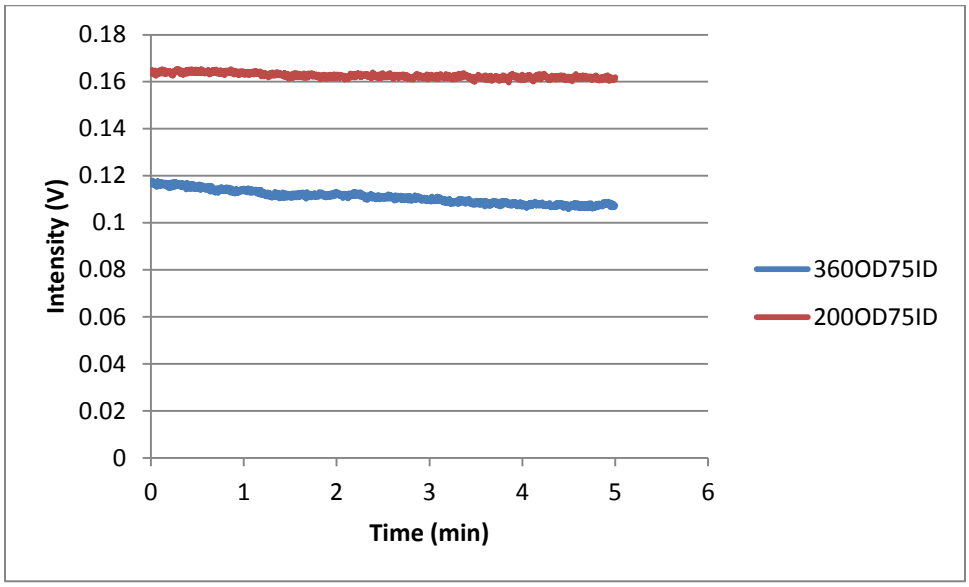

Figure 5.2. Fluorescence signal of 10 uM tryptophan in capillaries with 75 um inner diameter and varying outer diameter. Signal was measured after 5 minutes to allow photobleaching of the capillary silica and optical lenses. Background was calculated by measuring the fluorescence signal of deionized water and subtracted from the tryptophan signal. 200 um OD was found to yield the most intense fluorescence signal.

Figure 5.3

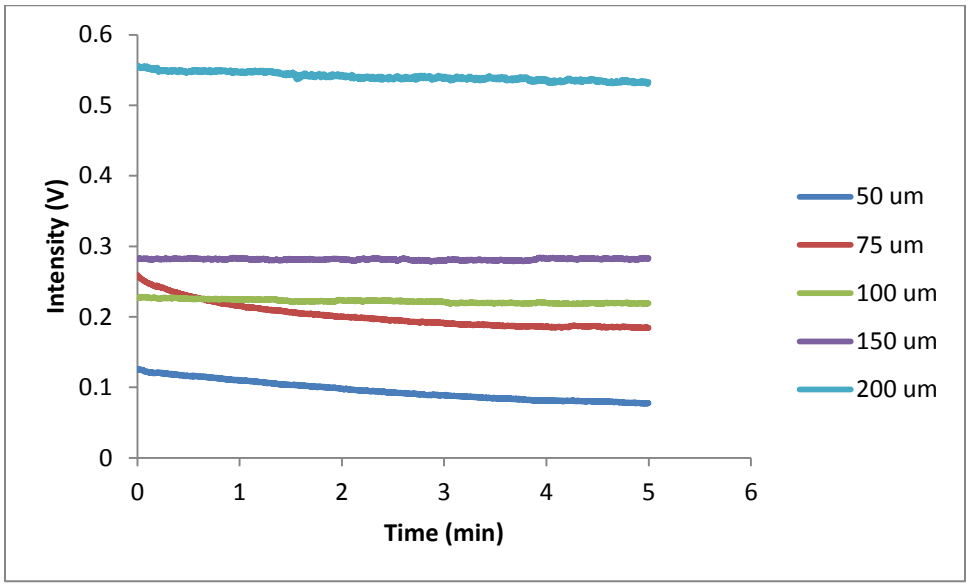

Figure 5.3. Fluorescence signal of 10 uM tryptophan in capillaries with 360 um outer diameter and varying inner diameter. 200 um ID was found to yield the most intense fluorescence signal.

Figure 5.4

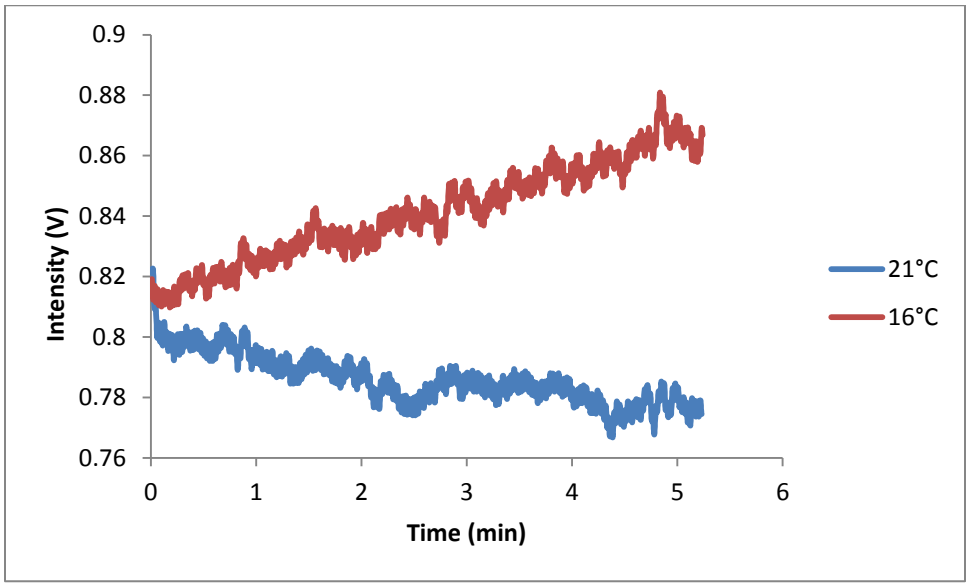

Figure 5.4. Fluorescence intensity of 10 uM tryptophan solution at different temperatures. A cold air gun was used to cool the LED to 16°C, upon which the LED was turned on and fluorescence intensity was measured by photomultiplier tube. After being allowed to acclimate for 5 minutes, the fluorescence intensity was found to be 7% higher at 16°C than at 21°C.

Figure 5.5

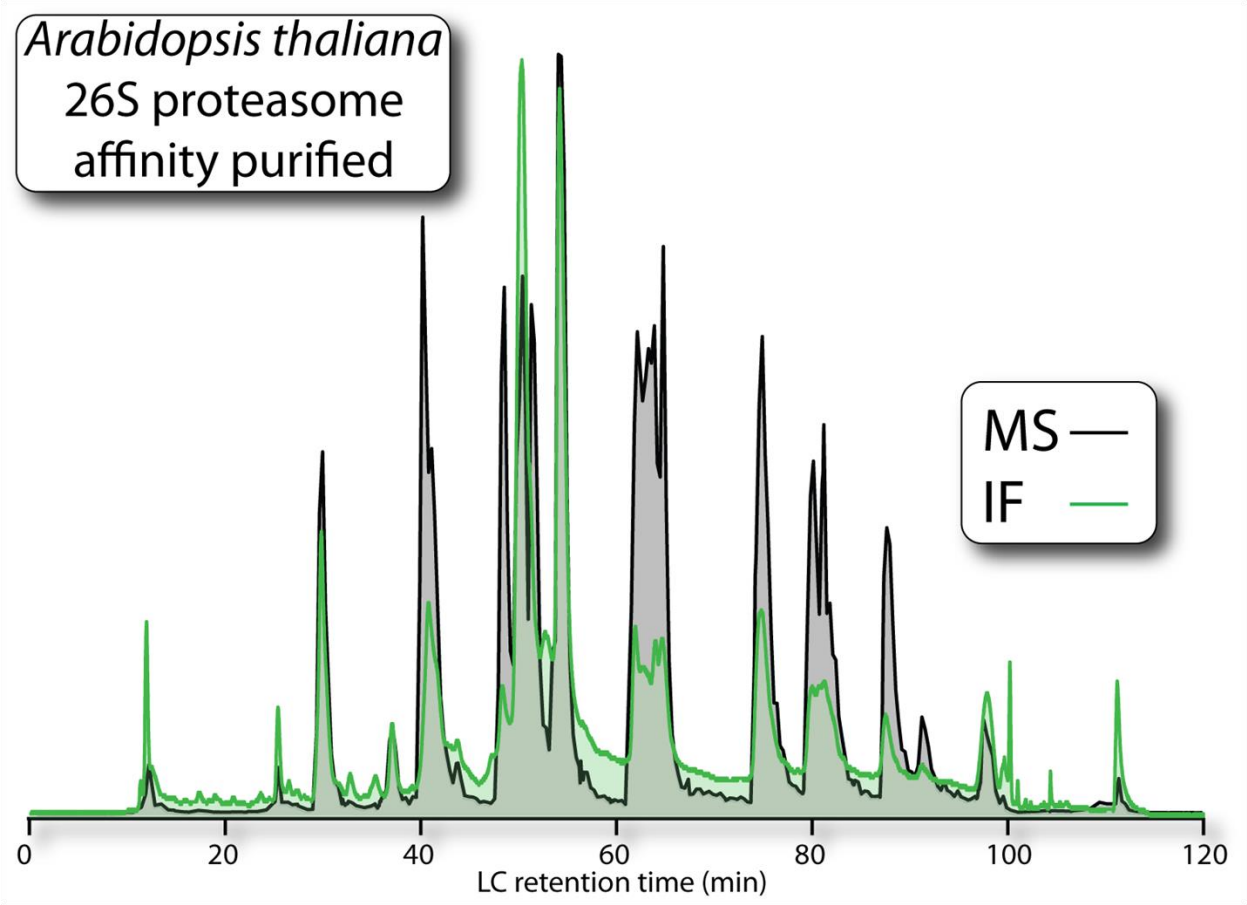

Figure 5.5. LED-induced native fluorescence chromatogram overlaid with mass spectrometric total ion chromatogram. 26 proteins were detected by mass spectrometry, and 13 tryptophan-containing proteins were detected by LED-induced fluorescence.[12]

Figure 5.6

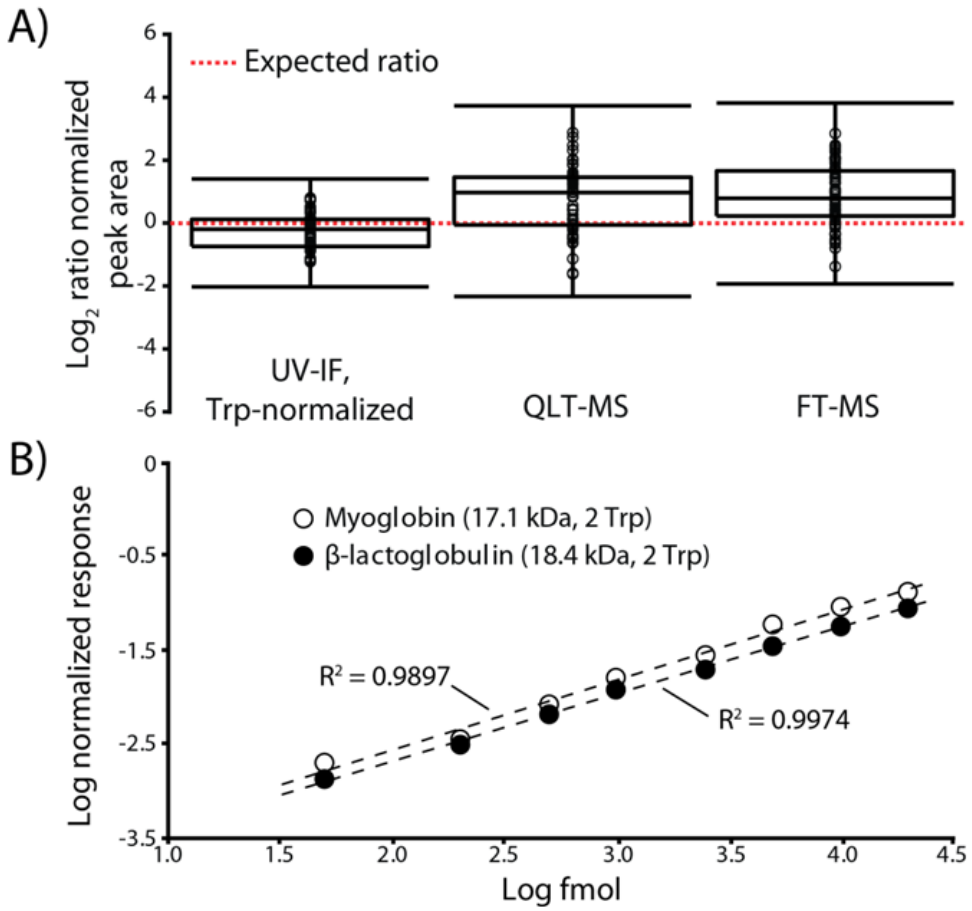

Figure 5.6. Accuracy, precision, and dynamic range of protein quantification by LED-induced fluorescence. The signal response (peak area) was normalized for each detection method and plotted on a log scale. A) Quantification by LED-induced fluorescence was found to be more accurate and more precise than quantification by mass spectrometric peak area. B) Fluorescence signal was found to have a linear relation to protein quantification over three orders of magnitude for myoglobin and ß-lactoglobulin.[12]