Chapter 2

Performing Comparative Peptidomics Analyses of *Salmonella* from Different Growth Conditions

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Abstract

Host–pathogen interactions are complex competitions during which both the host and the pathogen adapt rapidly to each other in order for one or the other to survive. *Salmonella enterica* serovar Typhimurium is a pathogen with a broad host range that causes a typhoid fever-like disease in mice and severe food poisoning in humans. The murine typhoid fever is a systemic infection in which *S. typhimurium* evades part of the immune system by replicating inside macrophages and other cells. The transition from a foodborne contaminant to an intracellular pathogen must occur rapidly in multiple, ordered steps in order for *S. typhimurium* to thrive within its host environment. Using *S. typhimurium* isolated from rich culture conditions and from conditions that mimic the hostile intracellular environment of the host cell, a native low molecular weight protein fraction, or peptidome, was enriched from cell lysates by precipitation of intact proteins with organic solvents. The enriched peptidome was analyzed by both LC–MS/MS and LC–MS-based methods, although several other methods are possible. Pre-fractionation of peptides allowed identification of small proteins and protein degradation products that would normally be overlooked. Comparison of peptides present in lysates prepared from *Salmonella* grown under different conditions provided a unique insight into cellular degradation processes as well as identification of novel peptides encoded in the genome but not annotated. The overall approach is detailed here as applied to *Salmonella* and is adaptable to a broad range of biological systems.

Key words: Comparative proteomics, *Salmonella*, mass spectrometry, peptide extraction, native proteases, accurate mass.

1. Introduction

Controlled and coordinated protein degradation is critical for biological systems to function properly. The processes of protein degradation have roles in the cell-cycle (e.g., cyclins), signaling cascades (e.g., receptor shedding), protein maturation (e.g.,

plasminogen), and nutrient cycling. Despite the critical roles of protein degradation in biological processes, there have been surprisingly few systematic global analyses of protein degradation; the majority of studies that have been performed focus on eukaryotic systems. Specific protein degradation processes are very highly regulated in bacteria and determined by environmental conditions. Selective degradation of proteins followed by cannibalization of the released amino acids is the most efficient process for bacterial adaptation to changing metabolic requirements (1, 2). Indeed, the ability of a pathogen to survive in the host and exploit new resources is an essential virulence trait.

The development of novel antibiotics against bacterial pathogens represents just a single discipline that can benefit from the elucidation of selective protein degradation processes. Recently our group developed an LC–MS/MS-based approach to globally profile a sub-set of peptides in a biological sample. Peptides, defined here, are short chains of amino acids linked via peptide bonds and are typically composed of fewer than 100 amino acids. The source of peptides in a biological system may result from short genes or through targeted degradation of proteins. Most of the peptides observed in this recent study were found to be the products of protein degradation (3); regardless of source we refer here to this naturally occurring peptide fraction as the "peptidome".

Interestingly, nearly 2% of the 4550 predicted proteins in *S.* typhimurium are annotated as being involved in protein degradation. Importantly, nearly all of these proteolytic proteins were identified in an early analysis of the *S.* typhimurium proteome, indicating that there is an upregulation of these functions under some of the growth conditions studied (4). The following is a step-by-step description of the sample preparation and analytical procedures that were used in determining the *Salmonella* peptidome. In addition, a discussion of the data analysis concerns that are unique to analyzing peptidomics samples is included.

2. Materials

Unless stated otherwise, Materials were obtained from Sigma Aldrich, St. Louis, MO.

2.1. Cell Growth and Isolation

Cellgro Dulbecco's Phosphate Buffered Saline (Mediatech, Mannasas, VA).

2.2. Lysis/Peptide Extraction Reagents

1. Water purified using a NANOpure® or equivalent system (≥ 18 MΩ×cm, Barnstead International, Dubuque, Iowa).

2. Ammonium bicarbonate, isopropanol, and methanol (Sigma Aldrich).

3. Protease inhibitor cocktail formulated for use with bacterial cell extracts (Cat. No. P8465, Sigma)

4. 0.1 mm zirconia/silica beads (BioSpec Products, Bartlesville, OK) were used for cell lysis.

5. 10–20% Tris-Tricine Ready Gels® (Bio-Rad, Hercules, CA) and GelCode® Blue reagent from Pierce for SDS-PAGE analyses.

6. OMIX® C-18 tips (100 μL) (Varian, Inc, Palo Alto, CA) for sample solid-phase extraction (SPE) clean-up prior to MS analysis.

7. SpeedVac (Thermo Fisher Scientific, Waltham, MA) to concentrate samples.

2.3. Liquid Chromatography– Mass Spectrometry/Mass Spectrometry

1. Ion trap mass spectrometers (LTQ, Thermo Fisher Scientific, San Jose, CA)

2. Water purified using a NANOpure® or equivalent system ($\geq 18 \text{ M}\Omega \times \text{cm}$)

3. Mobile phase A: Degassed 0.2% acetic acid, 0.05% trifluoroacetic acid in water (Sigma Aldrich)

4. Mobile phase B: Degassed 0.1% trifluoroacetic acid in 90% acetonitrile (ACN), 10% water (Sigma Aldrich)

5. 5-μm Jupiter C_{18} stationary phase (Phenomenex, Torrence, CA) packed into 60-cm (360 μm o.d. X 150 μm i.d.) fused silica capillary tubing (Polymicro Technologies Inc., Phoenix, AZ)

6. Liquid chromatography system is described elsewhere by Livesay et al. (15)

2.4. Liquid Chromatography– High-Resolution Mass Spectrometry

1. Fourier transform ion cyclotron resonance (FTICR) mass spectrometer, either a custom-built 11 T instrument or 9.4 T instrument (Bruker Daltonics, Billerica, MA).

2. *See* **Section 2.3** for details on mobile and stationary-phase materials.

2.5. LC–MS/MS Data Analyses

1. SEQUEST® version [TurboSEQUEST® (cluster) v.27 (rev. 12), Thermo Fisher Corp.]

2.6. Proteomics

1. RapiGest™ (Waters, Milford, MA) is a surfactant to aid in the solubilization and trypsin digestion of proteins.

2. Trypsin for protein digestion (Promega, Madison, WI)

3. Bicinchoninic Acid (BCA) Protein Assay kit (Pierce, Rock-ford, IL) for quantitation of peptides

2.7. Data Visualization and Cluster Analysis

1. *DAnTE*, freely available software for comparative analysis of proteomics data available at http://omics.pnl.gov/software/

2. *MultiExperiment Viewer* (MEV) is also freely available and designed for use in microarray experiments, but can be particularly useful for proteomics data visualization and clustering and is available at http://www.tm4.org/mev.html.

3. Methods

3.1. Culturing Conditions

The culturing conditions of the bacteria are not the focus of this review but are summarized here. The primary difference between the culture conditions used is that various forms of stresses, some relevant to pathogenesis, were compared relative to a rich growth medium at middle logarithmic growth phase. Wild-type *S.* typhimurium strains 14028 and LT2 were grown to mid-logarithmic (Log) and stationary (Stat) phases in Luria-Bertani (LB) broth and harvested for analysis. Two other cell growth conditions were used that differed only in the pre-growth conditions. In one, the bacteria were grown to stationary phase in LB, the bacteria were isolated, washed, and then grown in magnesium-minimal acidic medium (Shock); in the other, the bacteria were diluted 1:100 and grown in acidic minimal media overnight (Dilu). All cultures were harvested following standard batch culture techniques as outlined (*see* references (3–5) for more detail of culture methods). Aliquots of cell cultures (corresponding to 0.15 g cell pellets) were pelleted, washed in PBS, flash frozen with liquid N_2, and used as needed to prepare samples.

3.2. Sampling Preparation and Peptide Extraction

The procedures outlined here are specific to samples that require Biosafety Level 2 (BSL2) containment and treatment. Many of the precautions (e.g., O-ring sealed cryovials, cooling following vortexing) are to prevent aerosolization of unlysed pathogenic organisms. When developing these protocols, we lysed cells in the presence of a protease inhibitor cocktail formulated for use with bacterial cell extracts. However, we did not evaluate corresponding analyses without the protease inhibitor cocktail (*see* **Note 1**). The listing of class-specific chemical inhibitors of proteases found in the excellent review by Overall and Blobel (6) may be consulted if protease inhibition is desired. In our previous work, we

performed tests to mimic poor sample handling (incubation at 22°C for 20 min without inhibitors) and compared these results to those obtained when the samples were prepared at ∼7°C with a cooling block (normal handling temperatures with inhibitors) (*see* **Note 2**). We found no significant variation in the peptides identified. The procedure below is based on an isopropanol extraction that causes larger proteins to precipitate while endogenous peptides are maintained in solution. Different concentrations of isopropanol were tested and it was determined that a ratio of 3:2 resulted in the best recovery of endogenous peptides from *S. typhimurium*. This may not hold true for all biological samples.

1. Lysis of bacterial cells is accomplished by first resuspending the cell pellet in an equivalent volume of 100 mM NH_4HCO_3, followed by transfer of the sample to a 2.0-mL O-ring sealed cryovial. Next, 0.1-mm zirconia/silica beads are added to half of the volume in the tube, and the tube is then vortexed for 30 s, followed by cooling for 1 min in a cold-block. Six cycles of vortexing and cooling are performed. The lysate is then removed from the top of the settled beads, and the beads are rinsed five times with buffer. The lysates and rinses are then pooled separately in microcentrifuge tubes (*see* **Note 3**).

2. The pooled lysate is centrifuged at $16,000{\times}g$ for 10 min at room temperature to pellet insoluble and precipitated proteins. Transfer the supernatant to a new microcentrifuge tube, and ensure that the entire pellet is left behind. The supernatant is now considered a cleared lysate. An aliquot of the cleared lysate can be saved for SDS-PAGE as a reference.

3. Isopropanol is then added to the cleared lysate in an appropriate ratio (we used 1:1, 3:2, 2:1, or 5:2 (v/v, isopropanol:lysate)), and the samples were mixed by vortexing. Pre-cooling the isopropanol to 4°C before adding to the lysate can assist with precipitation of proteins. The samples are then incubated at 4°C for 15 min, then microcentrifuged at $16,000{\times}g$ for 10 min at 4°C to remove precipitated proteins. The resulting supernatants are transferred to new microcentrifuge tubes and concentrated in a SpeedVac to ∼75 μL. Ten-microliter aliquots can be removed at this time for SDS-PAGE.

4. Peptide concentrations are determined by BCA protein assay, and SDS-PAGE analyses are performed using 10–20% Tris-Tricine Ready Gels®. The Tris-Tricine gels are used because they are specific for the separation of extremely small proteins and peptides. Gels are fixed for 30 min in 40% methanol/10% acetic acid and then stained for 60 min using GelCode® Blue reagent.

5. Prior to MS analysis, the concentrated isopropanol extracts are cleaned via solid-phase extraction using OMIX C18 pipette tips. These tips are monolithic, rather than particulate, and are therefore much easier to use without clogging, while providing better recovery and reproducibility. Thirty micrograms of peptide mass from each sample is applied to a 100-μL tip. The directions provided by the manufacturer are used to condition, wash, and load the samples. Peptides are eluted from the tips with 80:20 ACN:H_2O containing 0.1% TFA. Eluted peptides are concentrated to ~15 μL in a SpeedVac.

6. Alternatively, samples can be fractionated using strong cation exchange (SCX) HPLC to minimize sample complexity prior to each LC–MS/MS analysis, as described previously (7). Each fractionation is performed using approximately 150 μg (peptide mass) of concentrated isopropanol extract, resulting in 25 fractions that are concentrated in a SpeedVac to dryness. The samples are then reconstituted in 25 mM NH_4HCO_3 to a volume appropriate for LC–MS/MS analysis.

3.3. Liquid Chromatography–Mass Spectrometry/Mass Spectrometry

Our analytical instrumentation consists of commercially available platforms [e.g., ion traps (LTQ from ThermoFisher) and FTICR–MS (BrukerDaltonics)] that are in-house modified to increase the sensitivity and throughput of the analyses. However, the below LC–MS(/MS) approaches can be applied at a reasonable level of quality with more generally available off-the-shelf instrumentation. LC–MS/MS analyses are useful for making identifications and for semi-quantitation based on "spectrum counting" techniques (4, 8–10). These analyses are also used to build a database of identified peptides annotated with determined reversed-phase elution times (11) and calculated masses. This database (also referred to as a mass and time tag lookup table) is used with results from the high-resolution MS analyses (**Section 3.4**) to increase throughput, perform label-free quantitation, and improve peptide-sampling methods in the MS experiment. This is a simplified description of the accurate mass and time (AMT)-tag process developed in our laboratory, which has been extensively discussed elsewhere (12–14).

1. The concentrated C18 SPE eluents from the peptide cleanup procedure and the SCX fractions are then analyzed by reversed-phase microcapillary HPLC (15) interfaced through nanoelectrospray ionization (nanoESI) to an ion trap mass spectrometer, as described previously (4). Briefly, the technique used in our laboratory entails gradient elution of peptides over 100 min using a 360 μm OD × 150 μm ID × 65 cm long capillary column packed with 5 μm Jupiter C18 particles.

2. For typical "bottom-up" proteomics experiments, in which the proteins are digested with trypsin, the charge states of peptides detected during LC–MS/MS are typically +2 and +3. Detected peptides are then fragmented using collision-induced dissociation. It should be noted that the peptides detected from the *S.* typhimurium endogenous peptidome include more +4 and +5 charge states than typically observed for other sample types. Due to the larger number of higher charged species, electron transfer dissociation may be considered for future analyses of the endogenous peptidome.

3.4. LC–MS Analyses

1. Concentrated C18 SPE eluents are also analyzed in our laboratory by reversed-phase microcapillary HPLC–nanoESI–FTICR–MS (11.5 T) (16). The same chromatographic platforms are used for LC–MS/MS analyses as is used with the FTICR–MS, and during analysis of multiple samples to be compared, the same chromatography column and electrospray emitter is preferred. This reduces the number of confounding variables during an experiment for downstream data analysis.

2. The analysis order for an experiment such as this needs to be addressed to minimize the effects of analysis time and possibility of carryover from highly abundant peptides. This is referred to as "randomized block design" and is meant to remove experimental nuisance factors that can obscure true differences between samples (*see* **Note 4**). These blocks typically contain one replicate for each experiment and the order of the analyses within a block is randomized.

3. Peptides from the LC–MS spectra are identified using the AMT tag approach (14), including any peptides with +4 and +5 charge states. The necessary software tools are publicly available (http://omics.pnl.gov). This approach uses the calculated mass and the observed normalized elution time (NET) of each filter-passing peptide identification (*see* **Section 3.5**) from the previous LC–MS/MS analyses to construct a reference database of AMT tags. Features from LC–MS analyses (i.e., *m/z* peaks deconvoluted of isotopic and charge state effects and then annotated by mass and NET) are matched (13) to AMT tags to identify peptides in a manner that results in roughly 5% false-positive identifications. For each protein, the sum of its peptide peak areas (NET vs. peak height) is used as a measure of the abundance of its fragments within the peptidome.

3.5. LC–MS/MS Data Analyses

Peptides can be identified using a number of different publicly available software packages.

1. In this example, we utilize *SEQUEST*® to search the resulting MS/MS spectra against the annotated *S. typhimurium* FASTA data file of proteins translated from genetic code provided by the J. Craig Venter Institute – formerly TIGR (4550 protein sequences, http://www.jcvi.org/) (17). These analyses used a standard parameter file with a peptide mass tolerance = 3, fragment ion tolerance = 0, and no amino acid modifications. Also, these analyses search for all possible peptide termini (i.e., not limited to only tryptic termini). Separate *SEQUEST*® searches that use the above FASTA data file but with scrambled amino acid sequences are performed in parallel to estimate the false discovery rate.

2. *SEQUEST*® generally returns multiple peptide identifications for each MS/MS spectrum and for each parent ion charge state. Therefore, for each MS/MS spectrum and for each parent ion charge state, only the peptide identification with the highest XCorr value (i.e., the "top ranked hit") is retained here.

3. Limiting false identification of peptides is an especially challenging issue for natively produced peptides because cleavage state (i.e., trypsin cleavage sites) is often used in making confident identifications. PeptideProphet (18) values are also not applicable because of a strong bias for "tryptic" peptides. The estimated percentage of false-positive peptide identifications can be defined as $\%FP_{est.} = 100\% \times$ (number of scrambled peptide identifications) / (number of normal peptide identifications) (19). $\%FP_{est.}$ should be calculated for each charge state, XCorr_Cutoff value (the minimum XCorr value requirement, which ranged from 1.5 to 5 in units of 0.02), and ΔCn_Cutoff value (i.e., the minimum ΔCn value requirement, which ranged from 0 to 0.4 in units of 0.005). In an effort to maximize identifications, a two-dimensional analysis of the XCorr_Cutoff and ΔCn_Cutoff is used for each parent ion charge state. This method is different from typical proteomics analyses in that it does not use a single ΔCn_Cutoff value.

4. The optimal XCorr_Cutoff and ΔCn_Cutoff values for each parent ion charge state (+1 to +5) was determined in our previous work to be 1.84 and 0.21 (+1), 2.1 and 0.21 (+2), 2.8 and 0.23 (+3), 3.56 and 0.265 (+4), and 4.16 and 0.22 (+5), respectively.

5. A rough measure of the abundance of each parent protein and its fragments within the peptidome can be attained using a spectrum counting (i.e., tallying of filter-passing peptide identifications) approach (20).

3.6. Comparison to Proteomics

Peptidomics data (samples acquired without digestion) should ideally be compared to proteomics data (samples acquired using typical bottom-up proteomics approaches including the use of trypsin) from the same source material. This comparison ensures that the peptidomics results are interpreted and can be compared with peptides resulting from abundant proteins being non-specifically degraded. We performed a proteomics analysis with the same starting sample material to that used in the peptidomics experiment (4). Briefly, proteins are isolated and digested as described in the protocol provided by Waters with the modification of 2.0% TFA rather than using concentrated HCL to adjust to a pH of 3.0. Acid incubation occurred at 37°C for 1 h to fully precipitate the RapiGest^{TM} surfactant. The samples are centrifuged in a microcentrifuge at full speed to pellet the RapiGest^{TM} and the supernatant is returned to neutral pH with NH_4OH to allow for digested peptide concentration determination by BCA protein assay.

The resultant peptides are then fractionated using strong SCX HPLC (7) into 25 fractions. A single unfractionated sample and the full set of 25 SCX fractions are then analyzed by reversed-phase LC–MS/MS. MS/MS spectra are searched using SEQUEST$^®$ and filtered to reduce false-positive peptide identifications (3, 4, 20).

3.7. Data Visualization and Cluster Analysis

The comparative interpretation of the identified proteins and peptides can present unique challenges. In the case of comparing environmentally induced changes in the *S. typhimurium* proteome and peptidome, one challenge is that many proteins are not commonly observed across all conditions. If one generates a matrix of protein/peptides (rows) by experimental conditions (column) populated with values of spectral observations or peak abundance measurements, the unobserved proteins/peptides are sometimes referred to as "missing data". The source of an unobserved species can be the result of either of the following: (1) its actual absence in a sample, (2) it is present, but below the detection limit of the mass spectrometer, or (3) the identification did not pass various quality thresholds used for confident peptide identifications. This results in a less than ideal direct application of statistical methods typically used for comparisons of high-throughput data (microarrays) such as an analysis of variance (ANOVA). For this reason, we typically try to combine the abundance values for all peptides from a source protein into a single representative protein abundance for comparison across conditions. This collapsing of peptide abundance to protein abundance is often referred to by us as "protein roll-up" (*see* **Note 5**). These protein values are then grouped by similar abundance profile

changes using methods such as a hierarchical clustering, which are common for microarray analysis comparisons. The comparative analyses of the peptide and protein abundances are enabled with the use of data mining tools that offer clustering and heatmap visualization of the matrix form of the experimental results, e.g., *DAnTE* (21), *OmniViz*® (22), or *MeV* (23). Some considerations that must be made when analyzing the data are listed below:
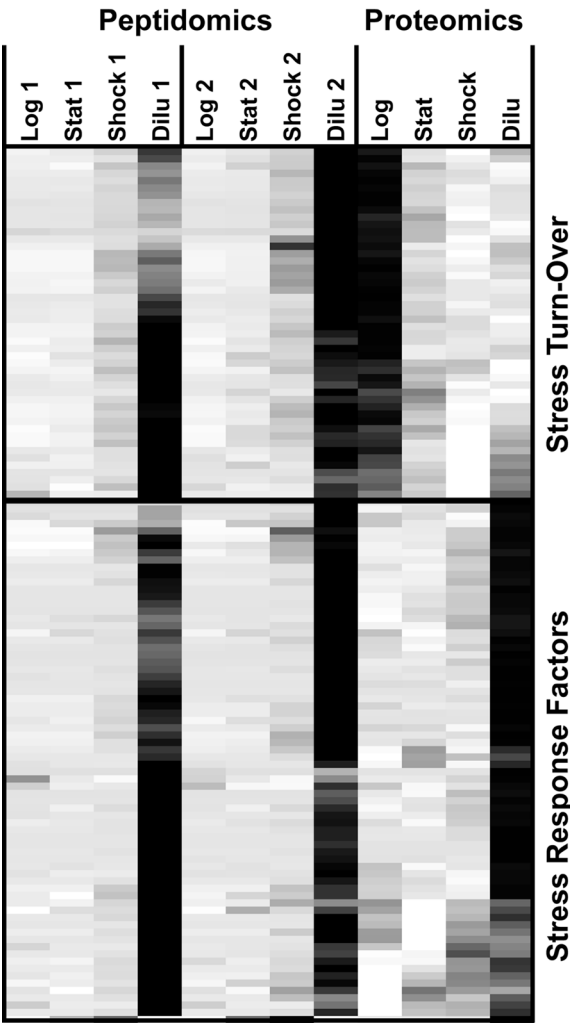

Fig. 2.1. Example heat map showing endogenous peptidomics results compared to global proteomics results. Observations across conditions were scaled using the Z-score across protein (with black representing a Z-score of 2.5 and white a Z-score of –1.0). Two selected regions were taken from data found elsewhere. "Stress response factors", in this case endogenously occurring peptides, correspond well with the abundance of the proteins in the proteomics experiments. The "stress turn-over" peptides appear to be scavenged in the "Dilu" stress condition, and these proteins appear to only be overly abundant in the rich logarithmic growth condition.

1. One of the first decisions is whether to fill arbitrary values into the unobserved peptide/protein abundances to make the analysis more amenable to various downstream data analysis methods typically applied in transcriptional microarray data analysis, such as ANOVA, principle component analysis, and/or clustering methods. If the number of spectra observed in a protein are used as a surrogate for an abundance measurement, filling might include applying the
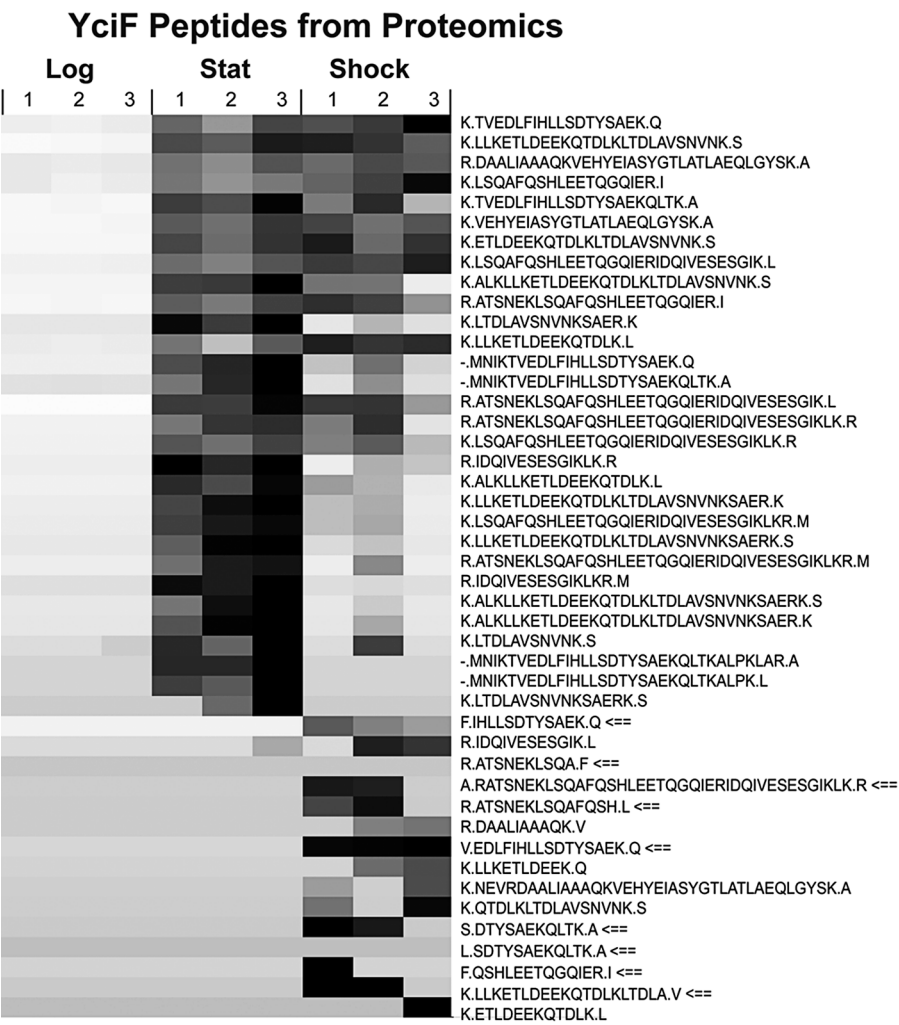

Fig. 2.2. A demonstration of proteomics results in the context of endogenous peptidomics. Although tryptic digestion was use in this example, the disappearance of a number of peptides between the stationary (stat) and shock conditions indicates that the protein is being differentially acted upon by proteases in the cell between the two conditions. This is especially true when this protein YciF was observed to be particularly abundant in the peptidome in the shock condition previously (3). As a secondary confirmation, new peptides that were not observed in the stationary condition appear in the shock condition. New "partially tryptic peptides" also appear and are highlighted with '<==' in the figure. The numbers under the conditions represent the biological replicate of that growth condition.

minimum number of required peptides for protein identification (*see* **Note 6**).

2. In both the peptide-centric and protein-centric (using a single abundance value for the protein) analysis, the difference in abundance between the most abundant peptide/protein versus the least abundant species may range several orders of magnitude. This large dynamic range of measurements may lead to difficulty comparing proteins with similar trends in a set of experiments. To use clustering tools, this dynamic range must be compensated for by scaling to similar magnitudes for comparison (i.e., a trend that is varied across 2 orders of magnitude should be grouped with other similar trends varying across 2 orders of magnitude even if the most abundant value to least abundant value between protein is across 6 orders of magnitude). Depending on the nature of quantitation (spectrum count versus peak area) and the number of experiments being compared (fewer than six versus thirty or more), different scaling approaches are preferred (*see* **Note 7**).

3. Once these steps are performed, comparisons between the experimental samples (both from the undigested native peptidome and the digested proteome) can be performed using heat maps of the clustered results (**Fig. 2.1**).

4. Once an endogenous peptidome analysis has been performed, and knowledge of proteins that are subject to native proteolysis is obtained, it is then possible to extract some additional information utilizing only a proteomics (i.e., trypsin was used) analysis by looking for non-tryptic cleavage sites (for an example **Fig. 2.2**).

4. Notes

1. It is reasonable to consider the goals of the experiment, there may be a specific desire to leave a class of proteases active to amplify the abundance of the cleaved products.

2. Set cooling block between 6 and 8°C, leaving the cooling blocks in the refrigerator 1 day prior to the experiment. Be sure to confirm that freezing will not occur by using microcentrifuge tubes of ~100 μL of water in the block during cooling.

3. All microcentrifuge tubes from this point forward should be siliconized (Fisher 02-681-332) to prevent polymer contamination, which is detrimental to downstream LC–MS(/MS) analyses.

4. The USA National Institute of Standards and Technology maintains an electronic *Engineering Statistics Handbook* (http://www.itl.nist.gov/div898/handbook) with a useful discussion of "Randomized block designs" for experiments.

5. "Protein roll-up" refers to methods that attempt to give a single value for each protein for quantitative purposes, even though each protein identification in a bottom-up proteomics experiment typically is based on more than one peptide identification. As of this writing, *DAnTE* offers multiple methods for protein roll-up (21).

6. Typically, an identification of a specific protein based on its tryptic cleavage products requires identification of three separate tryptic peptides. For native peptidomics this is not realistic because there is a high likelihood that only a single species will be present. Biological conclusions based on single peptide identifications should be based on methods with better relative abundance measurements such as the spectral peak abundance.

7. For large experiments, a Z-score (24) analysis can be helpful to visualize significant trends that are further than expected by a normal distribution. This is also better suited for peak area-based quantitation where the values are non-integers. For smaller experiments, dividing each value in a peptide or protein row by the associated sum, mean, or median of that entire row can be a useful method to scale the results.

Acknowledgments

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