A Comparative Study of In-gel Digestions Using Microwave and Pressure Accelerated Technologies

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Abstract

One of the most popular methods to prepare tryptic peptides for bottom-up proteomic analysis is in-gel digestion. To date, there have been few studies comparing various digestion protocols. In this study, we compare the efficiency of several popular in-gel digestion protocols along with new pieces of technology that may improve digestion efficiency, using a human epidermoid carcinoma cell lysate protein standard. The efficiency of each protocol will be based on the average number of proteins identified, their respective sequence coverage and relative quantitation using spectral counting. The importance of this study lies in its comparison of pre-existing in-gel digestion methods and newly developed technologies. These new technologies introduce the potential for a more cost effective digestion, higher protein yield and an overall reduction in time. The following four protocols will be compared: Shevchenko's overnight protocol (Methods in Molecular Biology 1999;122:383-397), a modification of the Modified Shevchenko's overnight protocol in which we removed the vacuum centrifugation steps, in-gel digestion in a barometric pressure cycler (Pressure Biosciences, Boston, MA), and in-gel digestion in a scientific microwave (CEM, Mathews, NC). In addition, several variables will be tested for increased digestion efficiency and keratin contamination including the elimination of vacuum centrifugation and the use of modified and unmodified trypsin. Statistical analysis will be performed on replicate samples to determine if there are any significant differences between protocols.

Digestion Methods

Sample Preparation

10 micrograms of the human epidermoid carcinoma cell lysate protein standard was run on a gel for 25 minutes, creating a gel blob. The gel blob, about 2 centimeters long, was fixed and stained overnight with Pierce's Imperial Protein Stain. The gel blobs were then cut into 1mm cubes and placed into 1.5mL tubes. The gel pieces were digested and extracted according to the following digestion methods using a modified, sequencing grade trypsin (Promega) and an unmodified trypsin (Wako). Four replicates of each digestion method were used.

Barocycler (Yellow)

The entire process takes place in a 150uL PCT Tube and Cap. Each cycle of the Barocycler digestion method increases the pressure to 20,000 PSI for 50 seconds, and then decreases the pressure to atmospheric pressure for 10 seconds. The entire digestion method, which takes about 40 minutes, consists of 40 cycles at 50 degrees Celsius. Each cycle of the Barocycler extraction method increases the pressure to 35,000 PSI for 10 seconds, and then decreases the pressure to atmospheric pressure for 10 seconds. The entire extraction method, which takes about 20 minutes, consists of 10 cycles at 50 degrees Celsius. Each cycle of the Barocycler reduction/alkylation method increases the pressure to 20,000 PSI for 50 seconds, and then decreases the pressure to atmospheric pressure for 10 seconds. The entire extraction method, which takes about 20 minutes, consists of 20 cycles at 50 degrees Celsius. In order to control for the pressure imparted by the Barocycler, we used a water bath digestion at 50 degrees Celsius where the reduction, alkylation, and digestion occurred in the non-pressurized water bath of the Barocycler at 50 degrees Celsius.

Microwave (Orange)

The Microwave uses the same method for the reduction, alkylation, digestion and extraction steps. During the entire method, the temperature is monitored by a fiber optic probe and held constant at 50 degrees Celsius. During the first cycle, the microwave power is increased to 50 watts for 2 minutes and 30 seconds. During the second cycle, the microwave power is decreased to 1 watt for 10 minutes. During the third and final cycle, the temperature is decreased to room temperature for 20 minutes.

Modified Shevchenko Method (Green)

Digestion: The gel pieces were washed twice with 50mM Ammonium Bicarbonate (AmBic), chemically dried twice using 100% Acetonitrile (ACN), and then mechanically dried using vacuum centrifugation. The gel pieces were reduced using 10mM Dithiothreitol in 100mM AmBic at 56 degrees Celsius for 30 minutes. The gel pieces were then alkylated using 55mM Iodoacetamide in 100mM AmBic at room temperature for 30 minutes. The gel pieces were washed twice with 50mM AmBic, chemically dried twice using 100% ACN, and then mechanically dried using vacuum centrifugation. Finally, 500ng of trypsin in 50mM AmBic was added and incubated overnight at 37 degrees Celsius.

Extraction: The supernatant was collected and put in a new tube. Next, 60% Acetonritrile with 0.1% Trifluoroacetic acid was added to the gel pieces. The gel pieces were sonicated for 10 minutes, centrifuged for eight minutes, and the supernatant was added to the previously collected supernatant. The supernatant was vacuum centrifuged.

Modifications

The following modifications were made to the previously described methods and labeled with a distinct color:

Blue – The Shevchenko Method was followed with the exception of the gel pieces being placed in the incubator overnight in a capped PCT microtube placed inside of a 1.5ml tube with 1mL of water to encourage a more constant temperature. The extraction step was performed in the Barocycler.

Cyan – The Shevchenko digestion method was followed and the extraction took place inside of the Barocycler. All vacuum centrifugation steps were removed and 40% ACN with 0.1% Trifluoroacetic acid was used for extraction. The samples were diluted to 5% ACN prior to loading.

Magenta – The Shevchenko Method was followed until the digestion step, which took place inside of the Barocycler. The Shevchenko extraction was employed.

Pink – The Barocycler Method was followed until the digestion step, which took place inside the incubator at 37 degrees Celsius overnight. The Barocycler extraction was employed.

Beige – The Barocycler Method was followed. All steps were performed in the non-pressurized water bath at 50 degrees Celsius rather than the pressurized chamber.

Purple – The Shevchenko Method was followed until the digestion step, which took place inside of the Microwave. The Modified Shevchenko extraction was employed.

Red - The Microwave Method was followed until the digestion step, which took place inside the incubator at 37 degrees Celsius overnight. The Microwave extraction was employed.

Rust – The Shevchenko Method was followed. All vacuum centrifugation steps were removed and 40% ACN with 0.1% Trifluoroacetic acid was used for extraction. The samples were diluted to 5% ACN prior to loading.

Figure1. Total Number of Unique Proteins Identified per Condition Using the Modified Trypsin

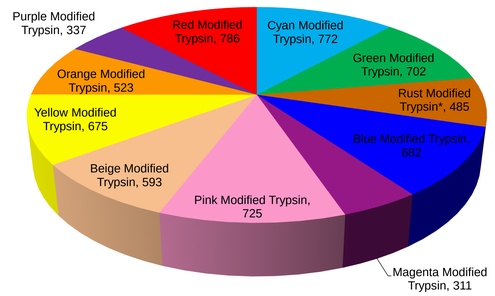


Figure 2. Total Number of Unique Proteins Identified per Condition Using the Unmodified Trypsin

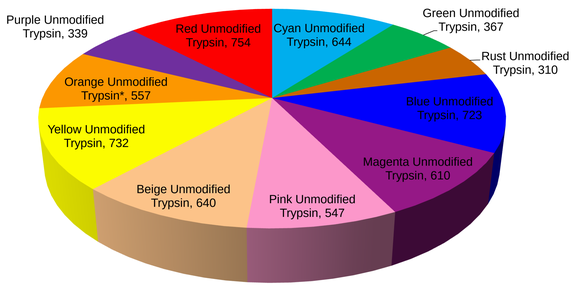


Figure 3. Average Number of Unique Keratin Peptides

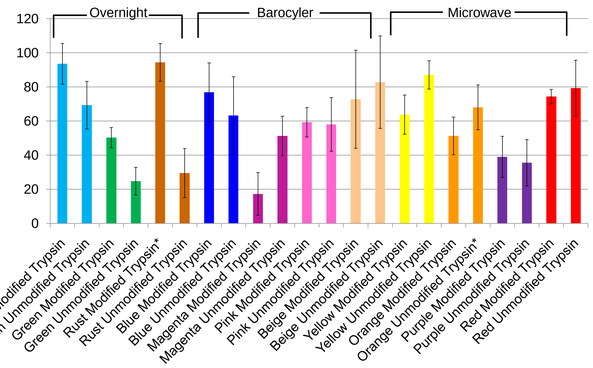


Figure 4. Average Sequence Coverage of 15 Differentially Expressed Proteins

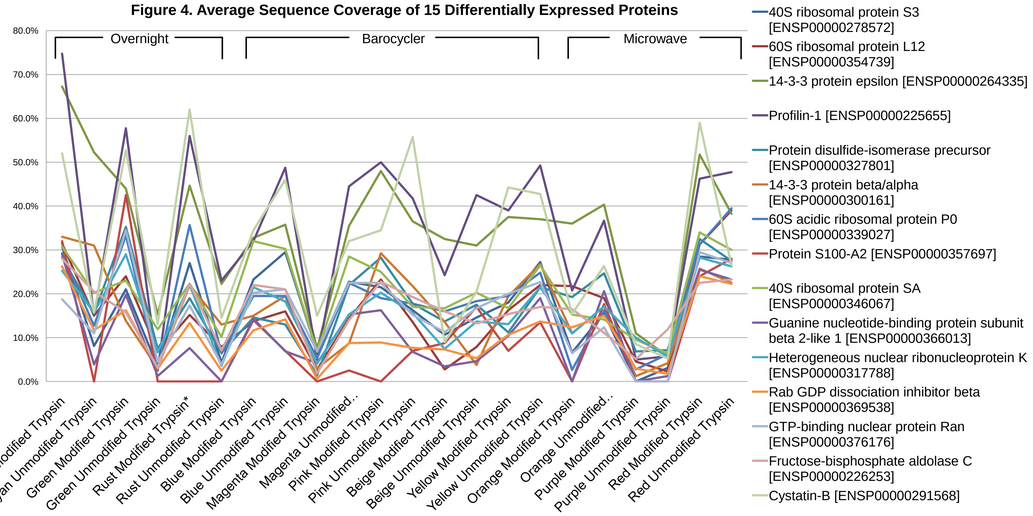
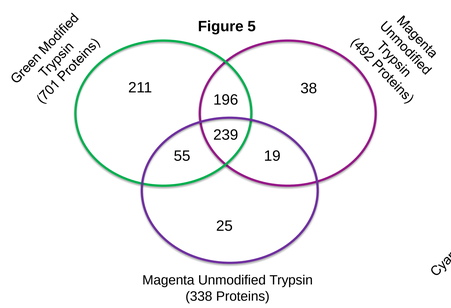


Figure1. The total number of unique proteins identified in each condition using the Modified Trypsin. **Figure 2.** The total number of unique proteins identified in each condition using the Unmodified Trypsin. **Figure 3.** The total number of unique keratin peptides identified in each condition. **Figure 4.** Average sequence coverage of 15 differentially expressed proteins with a P-value >0.000000001 for all four replicates across all conditions. **Figure 5.** Overlap of unique proteins identified in the 3 conditions using the Modified Trypsin (Trypsin was removed from the sample set). **Figure 6.** Average number of protein identifications for all four replicates across all 22 conditions. The bars in this figure represent plus and minus one standard deviation from the mean.

Figure 5



LC/MS Methods

The Paradigm MG4 HPLC System (Michrom Bio Resources, Auburn, CA) coupled with a Thermo Finnigan LTQ ion trap mass spectrometer (Thermo, San Jose, CA) was used for separation and analysis. 10 micrograms of each digested sample was loaded onto a trap column (Zorbax 300SB-C18, 5um, 5 x 0.3mm Agilent, Santa Clara, CA) and desalted. Peptides were then eluted from the trap, separated with a reverse-phase Michrom Magic C18AQ (200um x 150mm) capillary column at a flow rate of 2ul per minute. Peptides were eluted using a 90 minute gradient of 2% B to 35% B over 60 minutes, 35% B to 80% B for 3 minutes, held at 80% B for 2 minutes, 80% B to 5% B in 1 minute, and re-equilibrated for 8 minutes at 5%B (A = 0.1% Formic Acid, B= 100% Acetonitrile) and directly sprayed into the mass spectrometer. The mass spectrometer was operated with a spray voltage 1.8kV, a heated capillary temperature of 180ºC, and a full scan range of 350-1400 m/z. Data dependent MS/MS spectra were collected with the following parameters: 10 MS/MS spectra for the most intense ions from the full scan (minimum signal required = 500.0, isolation width = 2.0) with 35% collision energy for collision-induced dissociation. Dynamic exclusion of the same abundant peptides was enabled with a repeat count of 2 and an exclusion duration of 1 minute. Before each batch of samples run, BSA standards were run and scored using X!Tandem (minimum log(e) score of -500) in order to attempt to control for analytical variation.

Data Analysis

All MS/MS samples were analyzed using X! Tandem (The GPM, thegpm.org; version TORNADO (2008.02.01.2)). X! Tandem was set up to search null (unknown version, 47648 entries) assuming the digestion enzyme trypsin. X! Tandem was searched with a fragment ion mass tolerance of 0.40 Da and a parent ion tolerance of 1.8 Da. Iodoacetamide derivative of cysteine was specified in X! Tandem as a fixed modification. Deamidation of asparagine, oxidation of methionine, sulphone of methionine, tryptophan oxidation to formylkynurenin of tryptophan and acetylation of the n-terminus were specified in X! Tandem as variable modifications. Scaffold (version Scaffold_2_05_00, Proteome Software Inc., Portland, OR) was used to validate MS/MS based peptide and protein identifications. Peptide identifications were accepted if they could be established at greater than 80.0% probability as specified by the Peptide Prophet algorithm (Keller, A et al Anal. Chem. 2002;74(20):5383-92). Protein identifications were accepted if they could be established at greater than 80.0% probability and contained at least 2 identified peptides. Protein probabilities were assigned by the Protein Prophet algorithm (Nesvizhskii, Al Anal Chem. 2003 Sep 1;75(17):4646-58). Proteins that contained similar peptides and could not be differentiated based on MS/MS analysis alone were grouped to satisfy the principles of parsimony. *Two replicates, one from the Orange Unmodified Trypsin group and one from the Rust Modified Trypsin group, were removed from the sample pool because they were outside of at least 6 standard deviations from the mean.

Conclusions

Going into our experiment, our postulation was that the conditions that employed a short digestion (i.e., the Barocycler and the Microwave conditions), would yield a more efficient digest if the Unmodified Trypsin was utilized. This statement seemed to be true for the Barocycler condition (Modified Trypsin had an average number of 177 proteins identified, while the Unmodified Trypsin had an average number of 418 proteins identified), but not for the microwave condition (Modified Trypsin had an average number of 212 proteins identified, while the Unmodified Trypsin had an average number of 184 proteins identified).

Two conditions showed a marked increase in the number of proteins identified relative to the alternate condition. The standard overnight digestion using the Modified Trypsin had an average number of 487 proteins identified. The condition in which only the digestion took place in the Barocycler using the Unmodified Trypsin had an average number of 418 proteins identified. The condition in which only the digestion took place in the microwave using the Unmodified Trypsin had an average number of 184 proteins identified.

There was no discernible increase in the number of proteins identified when the extraction condition was varied and the digestion parameters were held constant. The standard overnight extraction yielded an average number of 487 proteins identified. The condition in which only the extraction took place in the Barocycler yielded 438 proteins identified using the Modified Trypsin and 513 proteins identified using the Unmodified Trypsin.

There was no discernible increase in the number of proteins identified when the vacuum centrifugation steps were removed from the standard overnight digestion. The standard overnight digestion with the Modified Trypsin yielded an average number of 487 proteins identified. The standard overnight digestion with the Modified Trypsin with the vacuum centrifugation steps removed yielded an average number of 444 proteins identified.

There was no discernible increase in the number of proteins identified when the entire process was carried out in the non-pressurized water bath as opposed to the pressurized chamber in the Barocycler. The Barocycler digestion with the Unmodified Trypsin yielded an average number of 424 proteins identified. The digestion with the Unmodified Trypsin that took place in the water bath at 50 degrees Celsius yielded an average number of 396 proteins identified.

More research must be done in order to validate these results since they are specific to this particular standard. We may come to find that the best approach would be to use a combination of the various technology platforms.

Future Experimentation

We plan to repeat this experiment using a simple protein standard such as Bovine Serum Albumin (BSA). The use of BSA will allow us to compare the sequence coverage of a single protein across all replicates without interferences of a complex matrix and also to determine whether or not we saturated the mass spectrometer detector. We also plan to repeat the experiment using another complex sample such as a bacterial or plant lysate to see whether the efficiency of each digestion condition was specific to this standard. We would also like to increase the gradient length of the LC-MS/MS run, in order to allow better separation and the possibility of identifying a larger number of proteins.

- ➢4 Non-analytical Replicates per Condition
- ➢10 Gels Run
- ➢44ug Trypsin Used
- ➢88 Total LC/-MS/MS Runs
- ➢176 Hours of Mass Spec Time
- ➢220 Hours of Labor to Perform the Digestions and Extractions
- ➢880ug of the Cell Lysate Standard Used
- ➢115,462 Total Unique Peptides Identified
- ➢2,393,555 Total MS/MS Spectra

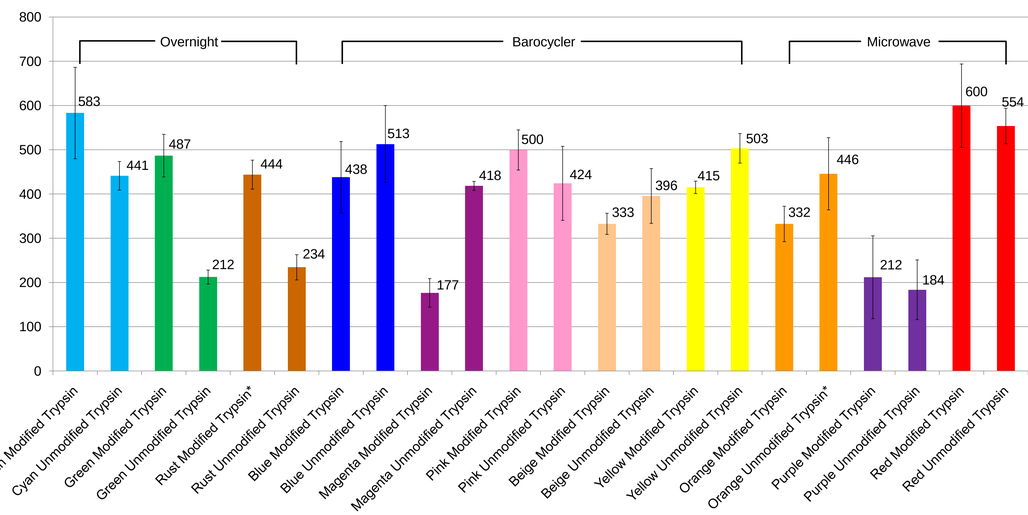



Figure 6. Average Number of Protein IDs