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Analysis of Peptide Quantification and Digestion through Assays, HPLC, and Modification of Workflow

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by

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Abstract

The goal of the project is to find a believable and accurate method to quantify peptides, by using a variety of commercial protein assays and HPLC. In the field of proteomics, mass spectrometry is the premier method for attaining the mass to charge ratio of a protein. Proteins must be applied to the mass spectrometer as digested peptide fragments. Quantification of peptide samples is therefore an important topic in both the preparation of samples to apply, as well as important information of unknown samples. Currently there is no method by which peptide concentration can be precisely found. Through the application and modification of already existing protein assays as well as HPLC methods, the sensitivity of the assay and technology for peptide samples will be measured. Methods in the workflow leading up to mass spectrometry must be considered as well such as purification of samples, robustness of methods to solubilize proteins and the methods by with proteins are digested.

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Introduction

Proteomics currently uses mass spectrometry as the major technique to find the mass-to-charge ratio of protein molecules. The samples that are applied to mass spectrometry are usually not intact proteins. The protein samples typically undergo trypsin digestion to create peptide fragments. Currently there is no definite method by which peptide fragment concentration can be determined. The quantification of peptides is thus an aspect of proteomics that is currently at the forefront of development with new products and techniques. Previously protein determination and concentration was at a similar point where reagents and techniques were being tested (Lowry et al 1951). This project attempts to find an accurate and believable method to quantify peptides produced from the digestion of proteins. Peptides have widely varying amino acid compositions and sizes and thus an accurate determination of concentration is difficult to manage.

Assay procedures generally use small volumes well within the range of 1-20 microliters. Currently peptide assays are being developed and released into the market, and this project aims to modify commercially available protein assays and gauge their effectiveness with peptide samples, as well as look at the sensitivity of the Pierce colorimetric and fluorometric peptide assay kits. There has been previous success with a modified bicinchoninic acid assay (Kapoor et al. 2009) in measuring peptide concentration that was examined as well. Ultimately by examining the accuracy and sensitivity of different methods, these results may be of use in accessibility and replication in other labs for the quantification of peptide samples.

The loss of, or contamination of, samples is a major factor to consider when optimizing workflow leading to mass spectrometry. For example a method that will be tested for the

robustness of digestion, versus the standard digest procedure, is the Perfinity Flash Digest by Perfinity Biosciences. The digest claims much faster digests with a smaller degree of human intervention which ultimately means lower contamination risks. Samples digested using this method will be compared to standard digested samples through the use of mass spectrometry and assay procedures.

A recent development in peptide analysis are assays that are directed at peptides such as the Pierce Quantitative Fluorometric Peptide Assay and Pierce Quantitative Colorimetric Peptide Assay. These kits will be gauged for effectiveness with a variety of samples versus changed assay protocols.

Methods

Homogenization Procedure and Mouse Sample Preparation

A random distribution of mouse organs was obtained and put into phosphate buffered saline (8g NaCl, 2g KCl, 1.4g Na_2HPO_4, 0.2g KH_2PO_4 in 800 mL dH_2O). These organs were segmented to approximately 2 mm sections with a scalpel blade on a clean glass plate in the laminar flow hood. These pieces were then homogenized with 2 mL RIPA buffer (0.25g Tris, 0.35g NaCl, 0.20g sodium deoxycholate, 0.04g SDS, 0.4mL Triton X-100 in 40 mL dH_2O) using a Potter-Elvehiem homogenizer. Samples were then sonicated in an ice water bath using pulses at 30% amplitude for 3 cycles. The reservoir of sample was then divided among 5 microcentrifuge tubes and centrifuged at 13000 rpm for 10 minutes at 19 degrees Celsius.

The supernatant was divided among three 2 mL centrifuge tubes and spun again at 13000 rpm for 10 minutes at 19 degrees Celsius. The supernatant was divided into 6 thick walled ultra

centrifuge tubes and spun at 50000 rpm for 1 hour at 16 degrees Celsius in the ultracentrifuge. Afterward the supernatant was removed to a clean 15 mL plastic tube and was stored at -20 degrees Celsius.

An undiluted sample, 1:4 dilution, and 1:10 dilution were analyzed via EZQ Assay and the concentration was found to be 58.66 ug/uL. The intact protein then underwent acetone precipitation and then was trypsin digested.

Precipitation Methods

Acetone Precipitation

A sample of known concentration which underwent acetone precipitation was needed in order to test digestion and peptide assay procedures. The proteins from several types of homogenates were used; these underwent acetone precipitation with the addition of four volumes of cold acetone (-20°C). The sample then underwent trypsin digestion with the following procedure. The acetone was removed from the pellet after being centrifuged. The acetone was then decanted off, the sample was washed three times with cold 4:1 acetone:water solution (-20°C) and centrifuged to restore the pellet. Afterward the sample was drained of the washing solution and placed in the speed vacuum until dry.

GE 2D Gel Clean-up Kit

Procedure B was followed which consisted of the following. Three volumes of precipitant solution was added to the protein sample and was incubated on ice for 15 minutes. Coprecipitant of three volumes was added to the sample and then vortexed. Tubes were centrifuged at 9200 rpm for 10 minutes. Supernatant was removed via pipette. This was repeated

again. Coprecipitant was added at a volume approximately 4x the volume of the pellet and the sample was centrifuged for 5 minutes. The supernatant was removed via pipette. Distilled water was added to cover pellet, and 1 mL of wash buffer was added, as well as 5 uL of wash additive. The solution was incubated at -20 degrees Celsius for 30 minutes and was vortexed every 10 minutes. Afterward the solutions were centrifuged at 9200 rpm for 10 min. The supernatant was removed and the pellet was left to air dry for 4 minutes.

Chloroform-Methanol Precipitation

A combination of 300 uL of pure water, 400 uL of methanol, and 100 uL of chloroform was added to 100 uL of sample containing 400 ug of protein (from mouse organ homogenate). The sample was vortexed and centrifuged at 13000 rpm for 10 minutes at 22 degrees Celsius. The methanol and water layer was removed without disturbing the interface. Then 400 uL methanol was added. The sample was then vortexed and centrifuged at the same settings again. Supernatant was removed and the sample was dried via speed vacuum. Finally the dried protein was rehydrated in 50 uL of pure water.

Digestion Procedures

Standard In-Solution Trypsin Digestion

20 uL of both ammonium bicarbonate and acetonitrile was added to an acetone precipitate of protein as well as to blank samples containing no protein. A solution of 10mM dithiothreitol was freshly made and 40 uL added to each sample and blank. The samples and blank were then incubated in a 60 degree Celsius water bath for ten minutes. Samples were then allowed to cool for fifteen minutes. A 55 mM iodoacetamide solution was freshly made and 20

uL of the solution was added to the samples and blanks. The samples and blanks were then incubated at room temperature for thirty-five minutes. Worthington trypsin was used for the digest and a 1:20 ratio of trypsin to protein was used. The trypsin solution consisted of trypsin in ammonium bicarbonate at a concentration of 1 ug/uL. The samples were then put into a 37 degree Celsius water bath overnight. After the incubation 20 uL of 0.1% formic acid was added.

Perfinity Flash Digest Protocol

The Perfinity Flash Digest is a trypsin digestion kit manufactured by Perfinity Biosciences. The kit features beads covalently linked to trypsin therefore reducing likelihood of autolysis. Samples were digested by combining the desired amount of protein with ultrapure water up to a volume of 50 uL. Then 150 uL of Perfinity Flash Digest Buffer was added. This solution was then transferred to a Perfinity Flash Digest PCR tube containing the covalently linked trypsin beads. The tubes were placed in a thermomixer which then incubated and agitated the samples at 70 degrees Celsius and 1400 rpm respectively for 75 minutes. Afterwards samples were either centrifuged and decanted from the beads, or C18 purification was done directly to the sample present in the tube.

Isolation of Peptides

C18 Purification

The filter tips that were used were NuTip Carbon-18 pipette tips. The binding and releasing solution used were formic acid (0.1%) and acetonitrile (70%) with formic acid (0.1%). Pipette tips were conditioned by aspirating and expelling 50 uL of releasing solution, and then washing the tip with three 50 uL aliquots of binding solution. Afterward the sample was bound

to the matrix by aspirating the sample twenty to fifty times. Samples were then washed by aspirating and expelling 50 uL aliquots of binding solution ten times. Samples were then released from the matrix by two aliquots of the releasing solution that were collected in a clean Eppendorf tube. To facilitate the release, the releasing solution was aspirated approximately ten to twenty times. The acetonitrile was then evaporated via speed vacuum and reconstituted to an equivalent volume of only formic acid (0.1%).

Assay Methods and HPLC

EZQ Assay

The EZQ assay is a commercially available protein assay by Invitrogen. Samples are spotted onto the EZQ filter paper included in the kit. After the spots are stained and washed, the fluorescence is measured in a scanner and compared to a standard curve. The EZQ assay was done with nitrocellulose as well as the included EZQ filter paper as nitrocellulose is commonly used in western blots and has a higher affinity for protein which was proposed to carry over to peptide quantification. The concentrations of methanol:dH2O used for the wash were a one-fifth dilution, one-half dilution and pure methanol. Other than these modifications the standard procedure of the EZQ Assay was followed as per the instructions in the kit. The assay was read through the use of a Bio-Rad ChemiDoc MP Scanner.

Quant-iT Protein Assay

The Quant-iT protein assay is a commercially available assay produced by Life Technologies. This assay was selected to be examined because the reagent and peptide sample

are contained in wells and do not undergo a wash, thus avoiding unwanted loss of the peptide sample The Quant-iT assay was used with no deviation from standard procedure of the assay. Volumes of samples that were applied to the wells of the microplate were 1 microliter. The assay was read at the standard 470/570 nanometer excitation and emission wavelengths using the florescence microplate reader.

BCA Protein Assay

The BCA protein assay is an assay commercially created by Thermo Scientific. The procedure was altered by the recommendation of Kapoor et al. (2009). These changes included having the samples and standards in 0.1M sodium hydroxide and 1% SDS. Samples were then incubated in a 95 degree water bath for 5 minutes and then allowed to cool back to room temperature. The final change was that after pipetting of samples, standards, buffer and reagent into designated wells, the microplate was encased in film, and incubated in a 37 degree water bath for 30 minutes for color development. Reading was done at an absorbance of 570 nm.

Pierce Quantitative Fluorometric Peptide Assay

Standards were created from serial dilutions of the stock standard solution included with the kit (1000 ug/mL). In a black microplate 10 uL of sample or standard was pipetted in triplicate. To each well 70 uL of assay buffer was added. Finally 20 uL of the assay reagent was added to each well. The microplate was then covered and incubated at room temperature for five minutes and then read at an excitation and emission of 390 nm and 475 nm respectively.

Pierce Quantitative Colorimetric Peptide Assay

Standards were created from serial dilutions of the stock standard solution included with the kit (100 ug/mL). In a clear 96 well microplate 20 uL of sample or standard was pipetted in triplicate. The working solution consisted of 50 parts of component A, 48 parts of component B and 2 parts of component C. 180 uL of working solution was added to each well. The microplate was then covered and incubated at room temperature for thirty minutes for color development and then read at an absorbance of 480 nm.

High-Performance Liquid Chromatography

The HPLC that was utilized was an HP 1100 from Agilent Technologies. The column that was used contained carbon 18 media. The column was stored in methanol. Binding was done in formic acid containing 2% acetonitrile. The release of peptide from the column was done using a gradient increasing the percentage of acetonitrile to 60% over the course of 40 to 100 minutes.

Results

Nitrocellulose EZQ Assay

The EZQ assay was changed, such that the standard EZQ assay paper was replaced with nitrocellulose due to the idea that nitrocellulose would have a better affinity for peptide fragments. After the washes with methanol of varying concentrations, results showed that spotted peptide samples were most likely washed off thus resulting in no fluorescence.

Thermo Scientific Quant-iT Assay

The Thermo Scientific Quant-iT assay features the sample, reagent, and buffer solutions contained in the respective wells of a 96 well microplate. Due to no wash step being needed, the stipulation was that there should not be any loss of peptide sample.

Sample Name	Concentration Known (ug/uL)	Mean Concentration	Digest-Trypsin (concentration from Blank)
BSA 0	0	0.027	
BSA 25	0.25	0.249	
BSA 50	0.5	0.499	
BSA 100	1	1.00267	
BSA 200	2	1.99867	
BSA 300	3	3.00033	
BSA 400	4	4.021	
BSA 500	5	4.671	
ABC Blank	0	0.07	
.5 OV	0.5	0.598	
1.0 OV	1	0.845	
1.5 OV	1.5	1.196	
2.5 OV	2.5	1.563	
5.0 OV	5	1.571	
0 OV Digest	0	0.138	0
.5 OV Digest	0.5	0.308	0.17
1 OV Digest	1	0.536	0.398
1.5 OV Digest	1.5	0.738	0.6
2.5 OV Digest	2.5	0.887	0.749
5 OV Digest	5	1.369	1.231

Table 1. Table of Quant-iT Assay values. Features bovine serum albumin standards (BSA), intact ovalbumin protein, and corresponding concentrations of digested ovalbumin.

Ultimately the Quant-iT assay did show detection but values appeared to "plateau" for the ovalbumin samples at 1.5 ug/uL. Thus the Quant-iT reagent is not suitable for peptide analysis and does not show a believable correlation between digested and undigested samples.

Degree of Deviation in Pierce Fluorometric Assay

This experiment was the among the first attempt at gauging the effectiveness of the Pierce Fluorometric Peptide Assay.

Sample	Concentration (ug/uL)
1 MH4 C18 Purified	.635
3 HLMR C18 Purified	.205

Table 2. Pierce Fluorometric Assay of 1MH4 and 3HLMR Eye Muscle Samples. Expected Concentration was 1 ug/uL. The values were much lower than expected leading to look at the solution in which the peptides were dissolved in which was the releasing solution after C18 purification (70% acetonitrile, 01% formic acid).

Acetonitrile as an Interfering Variable

This experiment was conducted to observe the degree of variance that occurs with acetonitrile present past the recommended threshold of the assay kit.

Standards (ug)	Mean Concentration		Samples	Mean Concentration
Blank	0.002		Promega 3HLMR	0.264
0.078 Standard	0.078		Promega 3HLMR no acetonitrile	0.566
0.156 Standard	0.156		Promega 5GLLR	0.173
0.313 Standard	0.309		Promega 5GLLR no acetonitrile	0.409
0.625 Standard	0.62		Promega Control	0.265
1.25 Standard	1.25		Promega Control no acetonitrile	0.641
2.5 Standard	2.5		Perfinity 3HLMR	0.058
5 Standard	5.01		Perfinity 3HLMR no acetonitrile	0.147
10 Standard	9.26		Perfinity 5GLLR	0.09
			Perfinity 5GLLR no acetonitrile	0.148
			Perfinity Control	0.074
			Perfinity Control no acetonitrile	0.043

Table 3. Pierce Fluorometric Peptide Assay with 3HLMR and 5GLLR eye muscle samples digested using Promega brand trypsin and Perfinity Flash Digests. Samples were featured

with or without acetonitrile being evaporated off, respectively. Expected Concentration was 1 ug/uL based on the 100 ug protein aliquots of original sample.

Acetonitrile causes a significant shift in detected fluorescence. Assay steps must be done after removal of acetonitrile via speed vacuum.

Perfinity Digest Time Trials / Experimentation with Formic Acid for Matrix Binding

This experiment was done to examine if the amount of digestion varied with increased digestion times for the Perfinity Flash Digest. Samples were purified a second time to quantify how much peptide sample remained in solution after the first purification.

Standards (ug)	Quantity Detected	Sample	Concentration
0	0.003	Perfinity Digest 75 minutes C18 Purified	0.903
0.078	0.078	Perfinity Digest 75 minutes C18 Purified Twice	0.782
0.156	0.152	Perfinity Digest 110 minutes C18 Purified	0.835
0.313	0.323	Perfinity Digest 110 minutes C18 Purified Twice	0.782
0.625	0.607	Perfinity Digest 150 minutes C18 Purified	0.891
1.25	1.441	Perfinity Digest 150 minutes C18 Purified Twice	0.781
2.5	2.503	Perfinity Digest 150 minutes C18 Purified Control	0.08
5	5.297	Perfinity Digest 150 minutes C18 Purified Twice Control	0.048
10	9.793		

Table 4. Pierce Fluorometric Assay of Mouse Homogenate Samples Perfinity digested for different lengths of time (From 75-150 minutes). Samples were C18 purified multiple times to observe removal of peptides from digested solutions.

A 75 minute digestion period was seen to be the most effective time period for the Perfinity Flash Digest. From the multiple purifications done, there is significant sample that is not being purified using the C18 NuTips.

Mass Spectrometry Results of Standard and Perfinity Digests

This experiment was done to observe the degree of keratin, a common contaminant, present in thesamples that were digested with the Perfinity Flash Digest versus the in-solution trypsin digest.

Protein	Acension Number	Molecular Weight	A	B	C	D
Cluster of Keratin, type II cytoskeletal 1 OS=Homo sapiens GN=KRT1 PE=1 SV=6 (K2C1_HUMAN)	K2C1_HUMAN [2]	66 kDa	0	0	15	22
Keratin, type II cytoskeletal 2 epidermal OS=Homo sapiens GN=KRT2 PE=1 SV=2	K22E_HUMAN	65 kDa	0	0	4	7
Keratin, type II cytoskeletal 6A OS=Homo sapiens GN=KRT6A PE=1 SV=3	K2C6A_HUMAN (+1)	60 kDa	0	0	2	2

Table 5. Distribution of Keratin Across Perfinity Digest of Eye Muscle (HLMR). (A), Perfinity Digest B with n-octyl-glucoside (B), n-octyl-glucoside traditional method blank (C), n-octyl-glucoside traditional method (D). Result Values are displayed with number of spectra found.

Overall the Perfinity Flash Digest showed no keratin present in the digested sample most likely due to the lower possibility of contamination from a lack of interaction such as repeated pipetting. The use of n-octyl glucoside to increase solubility did not have a detected effect on this.

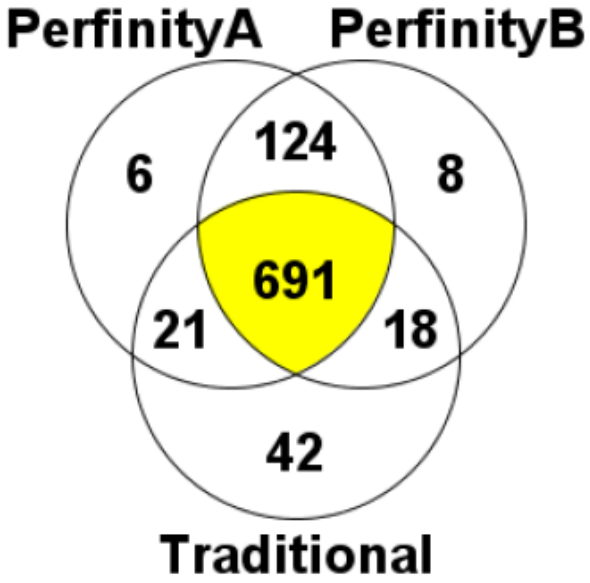

Figure 1. Venn Diagram of Distribution of Protein Species found Between Perfinity Digest and Traditional Digest

From this diagram there are certain species of protein detected that are specific to each digestion method. Thus there may be merit in doing both Perfinity Digest and in-solution digest prior to mass spectrometry. This is especially true for proteins that are not completely in solution for Perfinity Flash Digest.

Peptide Assay Analysis of Differing Digestion Methods Perfinity Flash Digest Vs Promega vs Worthington

This experiment was done to observe the differences in Perfinity Flash Digest versus in-solution digests done with two different brands of trypsin (Promega, Worthington).

Standards (ug)	Quantity Detected	Samples	Concentration
0	0.003	P3	0.405
0.078	0.107	P5	0.266
0.156	0.18	Pe3	0.115
0.313	0.284	Pe5	0.107
0.625	0.603	PeC	0.082
1.25	1.115	W3	0.368
2.5	2.64	W5	0.31
5	4.962	Standard Digest Control	0.053
10	10.03		

Table 6. Pierce Fluorometric Assay of 3HLMR and 5GLLR Eye Muscle Samples. Samples (1 ug/uL) were digested with the Perfinity Flash Digest (Pe), standard digest with Promega Brand Trypsin (P) or with the standard digest with Worthington brand trypsin (W). Samples were then C18 purified and assayed.

The Perfinity digest shows a lower concentration, but that may be due to a lack of contamination, or potential loss of sample during the purification step. Between the two different brands of trypsin used for the in-solution digest it can be observed that the values from the peptide fluorometric assay are similar showing the effectiveness of the digest regardless of the brand of trypsin.

BCA Assay on 2D Clean-up, and MeOH-Chloroform Precipitation

This experiment was done to observe the effectiveness of precipitation methods other than acetone precipitation prior to digestion.

Standard	Amount Detected (ug)	Sample	Mean Concentration (ug/uL)
0	0.051	2D Gel Cleanup	0.923
0.625	0.626	MeOH/Chloroform	0.875
3.125	3.112		
6.25	6.25		
12.5	12.6		
18.75	18.739		
25	25.002		
37.5	37.513		
50	46.491		

Table 7. Changed BCA Assay on Mouse Homogenate Protein Solubilized using GE 2D Clean-up, and MeOH-Chloroform Precipitation and Digested Using Perfinity Flash Digest. Concentration expected 4 ug/uL. Original sample of 400 ug.

The different precipitation methods were able to solubilize protein that was difficult to dissolve after acetone precipitation. The detected values was still not what was expected, showing that while the modified BCA assay does detect peptides being present, the degree of accuracy is questionable.

Colorimetric and Fluorometric Assay an Mouse Homogenate Samples

This experiment had two purposes. First to examine if the addition of 20 uL of 1% formic acid after Perfinity digestion would increase peptide binding to the C18 NuTip during C18 purification. Secondly, examining the variance between the colorimetric and fluorometric peptide assays.

	Peptide Fluorometric Assay (ug/uL)	Peptide Colorimetric Assay (ug/uL)
Mouse Homogenate Sample (No additions before C18 Purification)	21.81	37.72
Mouse Homogenate Sample (1% Formic Acid added prior to C18 Purification)	21.09	32.45

Table 8. Peptide Fluorometric and Colorimetric Assay Results of Mouse Homogenate Samples Treated with and without 1% Formic Acid Prior to Purification

Overall it appears that the addition of formic acid does not increase binding to the C18 NuTip. Additionally the colorimetric peptide assay values are much larger. This is most likely due to the color development necessary with the assay. In conclusion the peptide fluorometric assay appears to be the more accurate and believable method is estimating peptide concentration.

Discussion

EZQ Assay with Nitrocellulose Media

Adapting the EZQ protein assay to attempt to read a peptide sample did not yield a detected sample. This is most likely due to the methanol wash step removing the peptide molecules. Furthermore the affinity for both the reagent binding to peptides as well as peptides having similar affinity as proteins would for a nitrocellulose membrane were assumptions that had to be made during the conception of the experiment. This experiment showed that the peptide molecules applied to nitrocellulose in the EZQ protein assay did not bind well most likely due to their small size and were washed off the membrane during the wash step using methanol.

Quant-iT Protein Assay Applied to Peptides

The Quant-iT assay was chosen as a candidate for peptide quantitation due to the fact that assay reagents and peptide samples would all be contained in a microplate well. Upon observation of the Quant-iT assay results (Table 1), the general conclusion that was made was that the reagent that the kit uses was not effectively reacting with the peptide fragments, most likely due to the molecules being much smaller than the intact proteins. There was significant deviation among samples, and digest quantities appeared to plateau at higher concentrations.

GE 2D Gel Clean-up Kit and Chloroform Methanol Precipitation

The use of these methods to solubilize proteins did yield proteins completely in solution. The BCA assay on the 2D Gel Clean-up Kit and chloroform-methanol precipitated samples (Table 6) shows that peptides are being detected. The methods can be protocols that are utilized for less soluble proteins in the proteomic analysis workflow.

Altered BCA Assay from Kapoor et al. in Regards to Peptides

The altered bicinchoninic acid assay of Kapoor et al. does show significant color development over the standard protocol. Overall the protocol does function as the copper chelates between peptide bonds. The assay does lack in determining values accurately, and thus the workflow upstream sample analysis could be a confounding variable in analysis. The assay also involves a large degree of human interaction increasing the risk of contamination.

Perfinity Flash Digest

The Perfinity Flash digest is a very promising technique to efficiently digest protein samples in mass. Perhaps one of the best features is the relatively low degree of human interaction thereby reducing contamination (Table 4). The distribution of proteins that were found from the peptides present in the digest also differed versus the standard trypsin digest procedure. With this knowledge there may be benefit in using both methods with mass spectrometry for more comprehensive protein quantification. It should be noted as well that the 75 minute digestion time does create an optimized digestion. When the time of digestion was varied (Table 3) there was no significant difference in detected peptide quantities from the Pierce Fluorometric Peptide Assay Kit.

The separation of samples from the beads does create some difficultly without a vacuum manifold, as centrifuging and decanting the solution from the covalently linked trypsin beads risks a loss of sample. Overall performing the C18 purification in the Perfinity Flash Digest PCR tube has yielded no significant consequences. Another significant issue is solubility. The incidence of protein pellets in the flash digest creates a significant skewing of data as the pellet isn't removed during the digest procedure. Thus upstream workflow to solubilize particularly insoluble proteins may be deemed necessary for accurate digests.

Acetonitrile Interferes with Assay Reagents

When gauging the interference of acetonitrile in the fluorometric peptide assay (Table 2), it was determined that when acetonitrile was completely removed via speed vacuum, there was a significant change in detected peptide concentration. As samples that

are injected on the mass spectrometer are in a solution of 0.1% formic acid, it is gauged appropriate that peptide assay procedures should be done after the sample is reconstituted in 0.1% formic acid,, for both optimal column binding and reagent reactivity.

Perfinity Digest Versus Standard Protocol

When the standard and Perfinity digest methods were compared alongside the variation of trypsin (Table 5) it was determined that the digests are indeed producing peptide fragments. Deviation due to contamination was not gauged quantitatively but there is a degree of greater contamination in the standard digests upon comparison to the Perfinity digests and the corresponding controls. Interestingly enough the sequence grade Promega brand trypsin functions surprisingly similarly to the Worthington trypsin in terms of resulting peptide concentration produced.

HPLC Mediated Peptide Quantification

The use of the HPLC in peptide quantitation was not explored thoroughly due to an overall lack of sensitivity and accuracy. Originally the idea of excluding the solvent peak and integrating the remaining peptide peaks was sound mathematically, but in practice software limitations required manual peak analysis. The method can be elaborated on in the future, but from the testing that has been done so far, integration of HPLC peaks produced by peptides does not give an accurate representation of the peptide concentration of a solution.

Conclusion

The overall result of the experimentation revealed that the different assay techniques have various affinity for peptide samples. The EZQ Protein Assay, Thermo Scientific Quant-iT Protein Assay, and attempted quantification by HPLC do not work for peptides. The modified BCA Assay of Kapoor et al. does show sensitivity for peptide detection and can serve as a method to detect peptides. The recently released Pierce Colorimetric and Fluorometric Peptide Assay Kits appear to be sensitive and accurate to peptide concentration but further testing is necessary to ensure that workflow is optimized as there was a detected loss of sample when samples of known concentration were assayed. Between these two peptide assays, the fluorometric assay appears to be the more sensitive and accurate assay, as the colorimetric assay is dependent on a longer incubation for color development potentially leading to over development. Overall, from the experiments conducted, the Pierce Fluorometric Peptide Assay is the most reliable assay to analyze peptide samples.

The Perfinity Flash Digest is a potential addition to proteomic analysis due to its very short digestion time of 75 minutes. This method has a far lower probability of contamination due a lower degree of interaction versus the standard in-solution trypsin digest. Potentially both the Perfinity digest and in-solution digest can be used for more expansive protein detection in mass spectrometry. A particular obstacle would be the solubility of protein in the Perfinity Digest, which needs to be looked into as some protein samples are not as readily soluble. The 2D Gel Cleanup Kit by GE was able to solubilize

these proteins but may not be efficient for mass sample cleanup due to the extensiveness of the protocol. For the C18 purification of the sample, there is benefit to performing purification directly from the Perfinity Flash Digest PCR tubes as the C18 purification protocol wash step removes the trypsin bound beads. This is a positive alternative to centrifuging and decanting the peptide sample as the beads form an easily disturbed pellet.

There are still components of the workflow to consider for potential peptide sample loss. The C18 purification step appears to not remove all of the peptide from the sample, and thus requires further investigation. The addition of formic acid to increase the binding of peptides to the C18 matrix did not appear to have an effect (Figure 7).

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