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Extraction, Enrichment, Solubilization, and Digestion Techniques for Membrane Proteomics

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Abstract

The importance of membrane proteins in biological systems is indisputable; however, their amphipathic nature makes them difficult to analyze. In this study, the most popular techniques for extraction, enrichment, solubilization, and digestion are compared, resulting in an overall improved workflow for the insoluble portion of *Saccharomyces cerevisiae* cell lysate. Yeast cells were successfully lysed using a French press pressure cell at 20 000 psi, and resulting proteins were fractionated prior to digestion to reduce sample complexity. The proteins were best solubilized with the addition of ionic detergent sodium deoxycholate (1%) and through the application of high-frequency sonication prior to a tryptic digestion at 37 °C. Overall, the improved membrane proteomic workflow resulted in a 26% increase in membrane protein identifications for baker's yeast. In addition, more membrane protein identifications were unique to the improved protocol. When comparing membrane proteins that were identified in the improved protocol and the standard operating procedure (176 proteins), 93% of these proteins were present in greater abundance (higher intensity) when using the improved method.
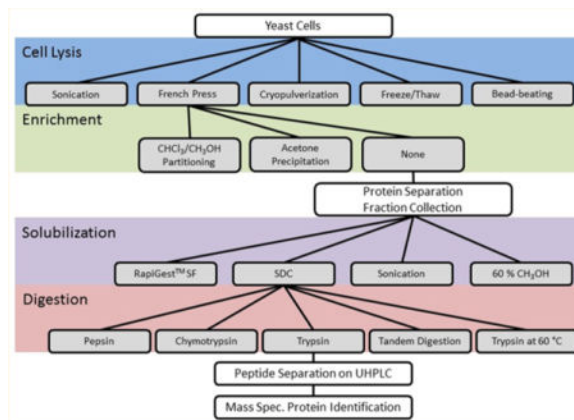
Graphical abstract

INTRODUCTION

Membrane proteins control biochemical processes, facilitate interactions between cells and their environment,[1–4] and are important pharmaceutical targets.[1,5–8] Although it has been widely recognized that membrane proteins provide critical functions within biological systems, the progress of their analysis has been slow compared with their soluble counterparts. This is predominantly due to their amphipathic quality, making membrane proteins extremely difficult to adequately extract, enrich, and solubilize from their native environment.[2,3,9–13] In addition to their amphipathic nature, membrane proteins are present in low levels,[1,4,5,13,14] increasing the difficulty of their detection in cell lysates. Despite the difficulties involved in their investigation, their important roles in biological processes and as drug targets make them an important focus of proteomic analyses.

In the field of proteomics, proteins can be analyzed intact (top-down proteomics), digested into peptides prior to analysis (bottom-up or shotgun approaches),[2,11] or a combination of both (such as prefractionation techniques).[15] Traditional shotgun approaches are ideal for membrane proteomics because the resulting peptides are easier to solubilize and separate than intact membrane proteins. While shotgun analyses are most common,[12] incorporating a prefractionation technique allows for an increase in protein identifications.[15–18] In our prefractionation approach, fractions are collected after a first-dimension chromatographic separation of intact proteins; these fractions are digested in solution, and the peptides are submitted to a second-dimension separation prior to mass spectral analysis. Prefractionation, that is, collecting fractions from a single chromatographic dimension and analyzing them on a second dimension, reduces sample complexity, increases overall peak capacity of the separation, and allows a greater amount of sample to be loaded for the possibility of finding low abundance proteins (such as membrane proteins).[4,15,19,20]

Although LC–MS plays a central role in proteomics, optimized sample preparation is required.[1,2,7] The sample preparation for membrane proteins typically encompasses extraction via cell lysis, enrichment, solubilization, and digestion steps for intact proteins, with the goal for these steps to be performed efficiently, without sample loss and residual interferences.[21] Common methods of protein extraction from the cell membrane include performing freeze/thaw cycles, cryopulverization, glass bead-beating, high-frequency sonication, and pressure-assisted (French press cell). Once the insoluble portion of the cell lysate is collected, an enrichment process can be used to isolate membrane proteins from contaminates generally collected along with membrane proteins. Chloroform/methanol partitioning[22–24] and acetone precipitation are the most common forms of delipidation[12,25] and are typically performed to avoid decreased performance in chromatographic separations and ion suppression in the mass spectrometer.[5,26,27] Chloroform/methanol partitioning works by providing an aqueous layer for soluble proteins, an organic layer to attract lipids, and an amphipathic interface to isolate membrane proteins. This technique works even in high concentrations of detergents, yet membrane proteins have been shown to disperse into multiple layers.[24,28] Acetone precipitation involves the addition of cold acetone to a buffered protein sample, causing the proteins to precipitate leaving the lipids, salts, and other small molecules in the acetone solution for elimination. Studies have shown, however, that a significant amount of lipids can remain with the sample after this procedure.[11]

Enriching the membrane proteins from the lipid bilayer and attempting to resolubilize them into an aqueous environment for analysis could cause protein precipitation or the formation of hydrophobic aggregates. Therefore, most membrane protein solubilization techniques utilize detergents (0.1–10% w/w) to mimic the lipid membrane,[4,5,7,14] but the performance of detergents is variable.[1,7,14,21] Also, the detergent must be removed prior to analysis because it can affect chromatographic separation and suppress ionization in the MS.[5,10,12,20,26,27] The acid-labile surfactant RapiGest SF (Waters) has been shown to greatly enhance membrane protein solubility[3] and allow 100% enzyme digestion at surfactant concentrations of 0.1% and can be easily removed prior to MS analysis via acidification.[29] The acid-insoluble detergent sodium deoxycholate (SDC) has also been reported to improve protein solubility and retain trypsin digestion efficiency at extremely high concentrations[9,10,21,30] (with 10% SDC, trypsin retained 77.4% activity),[31] and SDC can be removed prior to MS analysis via acidification or by ethyl acetate phase transfer.[4,30,32] Many other common surfactants, such as sodium dodecyl sulfate (SDS), can be utilized; however, most require thorough removal via dialysis or chromatography techniques, which can be problematic for high-throughput analyses and low abundance proteins.[10,13,33,34] SDS is a traditional surfactant that performs extremely well solubilizing membrane proteins, yet it is difficult to remove from the sample prior to LC–MS and can deactivate digestion enzymes.[10,13,35,36] One popular method utilizing SDS is FASP or filter-aided sample preparation.[37,55] This method was previously performed in the lab for shotgun proteomic samples (Figure S-1); however, results were inferior to in-solution digestions. Therefore, this method was not further investigated.

Either alone or in combination with detergents, organic solvents are often employed to aid in membrane protein solubilization.[12,13,35,38–41] The most common organic additive is ~60% methanol. Methanol dominates as the organic solvent of choice because of its amphipathic

nature as it induces lipid bilayer swelling and dissolution of membrane proteins.[39,42,43] Another less common solubilization technique during the sample preparation process is the use of sonication.[6,26,42] By applying sonication, hydrophobic vesicles can be dispersed, potentially releasing membrane protein clusters and allowing them to better interact with detergents (and therefore digestion enzymes) in solution.

As for the digestion itself, there have been reports of increasing protein coverage by using alternative protein digestion enzymes and applying tandem digestion techniques. With portions of the proteome difficult to reach with a single protease, various proteases can be used either individually or in tandem with trypsin to increase protein identifications and sequence coverage compared with a single protease.[4,13,44–46] For example, Swaney et al. discovered that by using multiple proteases for *S. cerevisiae* they achieved an additional 595 protein identifications and a 3-fold increase in sequence coverage.[46] The standard digestion protease is trypsin, cleaving at the carboxy-terminal of lysine and arginine residues. Sequencing grade trypsin can be easily acquired, from bovine or porcine sources, and is mainly used due to its reasonable specificity and stable activity.[20] Chymotrypsin is less specific; however, it cleaves proteins at more hydrophobic amino acids (such as phenylalanine and tryptophan)[13] and therefore may be a useful enzyme for membrane protein analysis. Nonspecific enzymes (such as pepsin) have also been studied, with the expectation that the production of overlapping peptides could increase protein sequence coverage;[47,48] however, this typically results in increased sample complexity for analysis.[45,46]

It is well established in the literature that sample preparation is extremely important (if not the most important) step in proteomic data acquisition.[2,8] This is especially true for membrane proteins that require careful preparation to avoid precipitation and aggregation for adequate digestion and supplemental identification. Although interest in the field of membrane proteins has increased, detailed and comparative sample preparation protocols are scarce. Typically, descriptions in the literature provide specialized protocols for the isolation of a specific type of membrane protein, compare relatively few methods, or focus on a specific aspect of the sample preparation process (cell lysis, enrichment, solubilization, or digestion). The comparison is further complicated because authors rarely use the same sample or instrumental methods across various studies.[4] The goal of this study was to investigate the major techniques of membrane protein sample preparation and determine a simple and efficient protocol that can be applied to increase the number of membrane protein identifications for the model organism *S. cerevisiae*. The techniques were required to be reproducible, discourage sample loss, and promote high-throughput analysis. As depicted in Figure 1, cell lysis, enrichment, solubilization, and digestion techniques were compared to determine an improved protocol for the analysis of membrane proteins.

EXPERIMENTAL SECTION

The experimental design is separated into four categories: cell lysis, enrichment, solubilization, and digestion, as shown in Figure 1. Each category was tested sequentially in the order previously listed. The sample preparation techniques within each category were compared to determine which sample preparation method proved paramount. Once a

technique was selected, the subsequent comparison within the next category utilized sample prepared from the optimized technique from the previous category. Prior to determining which sample preparation technique proved optimal, a default protocol was followed in the interim. For example, an optimal solubilization or digestion strategy had not been determined when comparing the enrichment methods. Therefore, as the enrichment methods were varied, the solubilization and digestion steps that followed were performed according to the default protocol until the next category was consecutively optimized. This resulted in changing only one variable in the protocol at a time while moving through the workflow shown in Figure 1. For the purposes of performing comparisons, the standard operating procedure (SOP) for our laboratory was chosen for comparison as it was previously followed in the lab and provided an acceptable general procedure to optimize specifically for membrane protein sample analysis. The SOP involved French press cell lysis, no enrichment, solubilization with 0.1% RapiGest, and digestion with trypsin at 37 °C. The solubilization and digestion steps[49] are described in detail in Figure S-2. Once optimal techniques were selected from all four experimental categories (cell lysis, enrichment, solubilization, and digestion) the completed improved protocol was compared with the default protocol in its entirety.

Cell Lysis

Saccharomyces cerevisiae (strain BY4741) was grown on glucose media until stationary phase was achieved (O.D. > 2). Cells were washed and resuspended in 50 mM ammonium bicarbonate, and samples were divided evenly (~25 mL) for cell lysis comparison. Sample that was not used for this study was flash-frozen (−80 °C) until the appropriate method of cell lysis was determined. When each lysis method was performed, protease inhibitors (Pierce Protease Inhibitor Mini Tablets, 88665) were added and prepared to the manufacturer's recommendations. In total, five cell lyses techniques were compared for yeast protein extraction (Figure 1): freeze/thaw, cryopulverization, bead-beating, sonication, and French press cell lysis. (1) For the freeze/thaw cell lysis, a 25 mL sample was allowed to freeze/thaw through three cycles reaching room temperature (37 °C) and returning to −80 °C without flash freezing to promote crystal lysis of cells. (2) Cryopulverization was conducted by pouring liquid nitrogen over a 25 mL sample in a mortar while grinding the sample into a powder using the pestle. The addition of liquid nitrogen was repeated to avoid thawing of the sample while grinding. (3) Another sample (25 mL) was placed in a centrifuge tube along with an equal volume of acid-washed 150–200 μm glass beads (Sigma, Type III glass beads, no. G-5255). The mixture was vortexed for 30 s periods for a total of 5 min and placed on an ice bath for 2 min in between bead-beating. (4) High-frequency sonication (Fischer Scientific Sonic Dismembrator Model 500) was performed on two 12.5 mL volumes (to avoid pulsation out of the 50 mL centrifuge tube) using a 1/8 sonication probe for three cycles (10 s of sonication at 30% power, followed by 2 min of incubation on ice). (5) Pressure-assisted cell lysis utilized a French press cell, where the entire 25 mL sample was passed dropwise at 20 000 psi three times through the cell. The cell itself was chilled (4 °C) and elutant was kept on ice throughout the process.

Each lysis sample was centrifuged at ~1200*g* for 10 min at 4 °C min to remove unbroken cells (Beckman, L8-70 ultra-centrifuge). The supernatant was isolated and underwent

ultracentrifugation at ~120 000g (38 00 rpm Beckman 60Ti rotor) for 90 min at 4 °C twice before the pellets (insoluble portions) were collected. To determine the amount of protein present, we performed a Bradford assay[50] (Coomassie Protein Assay Kit, Thermo Scientific) once the pelleted cells were resuspended in 50 mM ammonium bicarbonate buffer pH 8. The resulting concentrations were balanced through the addition of buffer, and 100 μg of each sample was enzymatically digested according to the digestion protocol.[49] The resulting peptides were processed on the modified ultra-high-pressure liquid chromatography (UHPLC) mass spectrometry (MS) system described later. Once French press cell lysis was determined to be the optimal method, it was performed (along with the ultracentrifugation steps previously described) to prepare the remaining sample for all further sample preparation techniques tested.

Enrichment

The two methods compared for the enrichment of membrane proteins were acetone precipitation and chloroform/methanol partitioning. These were compared with using no enrichment technique prior to the injection of intact proteins onto the first-dimension separation column (Figure 1). The acetone was precooled (−20 °C) for at least 1 h prior to use, and 0.5 mL was added to 1 mL of 0.5 mg/mL membrane protein solution (in pH 7.8 ammonium bicarbonate buffer). After acetone was added to these samples, the solution was vortexed and allowed to sit for 1 h at 4 °C. The sample was then centrifuged at 15 000 rpm for 5 min at 4 °C, and the supernatant was removed. The resulting pellet was dried under nitrogen and resuspended in buffer, and 80% formic acid was added immediately prior to intact protein separation. The formic acid solution is highly concentrated and should be handled with care.

For chloroform/methanol partitioning,[25] 1 mL of 0.5 mg/mL membrane protein solution was added to 4 mL of methanol (vortexed, centrifuged for 30 s at 9000g). Two mL of chloroform was added (vortexed, centrifuged 2 min), followed by 3 mL of water (vortexed, centrifuged 2 min). The water layer was then removed, and another 1 mL of methanol was added (vortexed, centrifuged 5 min). The sample was then centrifuged for 2 min at 9000g to pellet the protein. The chloroform/methanol supernatant was removed, and the remaining layers were dried under nitrogen and resuspended in buffer with 80% formic acid prior to injection onto the first-dimension protein-separation column. The injection onto the first dimension for the chloroform/methanol-enriched sample required four separate injections (0.250 mL each) of this particular sample. This had to be done as separate tandem injections loaded onto the head of the column with gradient starting conditions to avoid this technique clogging system tubing. Throughout the injection process the sample was kept chilled (10–15 °C) to avoid degradation.

Protein Separation and Fractionation

For the first-dimension protein separation, intact membrane proteins were placed in 80% formic acid (MS grade, Fluka Analytical) and injected onto a PLRP-S (300 Å, 5 μm, 250 × 4.6 mm, Agilent Technologies) polymeric reverse-phase column at 80 °C. The sample was not allowed to sit idle for >2 min in formic acid to avoid protein formylation (+28 Da)[11] prior to separation. Mobile phase A consisted of 80% water, 10% isopropanol, 10%

acetonitrile, and 0.1% trifluoroacetic acid (TFA). Mobile phase B consisted of 50% isopropanol, 50% acetonitrile, and 0.1% TFA. Beginning at 10% B, the gradient ramped to 60% B in 60 min at 1 mL/min, followed by a further increase to 90% at 65 min, where it held for 5 min before returning and stabilizing at 10% B. The total run time was 80 min to allow for re-equilibration. The progress of elution was monitored via UV detection (Waters CapLC2487, Waters, Milford, MA) at 214 nm, and 1 min wide fractions were collected for the 60 min gradient. Once the fractions were collected they were flash-frozen and lyophilized to concentrate the protein sample and remove MS-incompatible solvents. The fractions collected at 1 min intervals did not contain equal protein mass. It has been shown that dividing the sample into even amounts of protein resulted in more appropriate loading of the second-dimension separation and a higher number of protein identifications.[51,52] Therefore, the 60 lyophilized fractions were recombined into 10 fractions of equal protein concentration. The protein concentration was determined by integration of the UV absorbance signal collected during fractionation (Figure 2).[52] The fractions were then solubilized and digested[49] using the various techniques described later. This process was, of course, completed multiple times to individually test each enrichment, solubilization, and digestion technique.

Solubilization

A large amount (2 mg) of protein (without prior enrichment) was loaded onto the first-dimension protein separation (PLRP-S) column, and the resulting fractions were lyophilized and recombined into 10 equal mass fractions as previously described. These ten fractions were then further split into four equal sample sets (of 10 fractions each) to equitably compare solubilization techniques (Figure 1). Therefore, each solubilization technique was tested on a set of 10 fractions that had originated from the same first-dimension protein separation to reduce sample variability.

First, the application of detergents 1% sodium deoxycholate (SDC) and RapiGest SF was investigated. RapiGest SF acid-labile surfactant (ALS) (Waters) at 0.15% w/v was used to solubilize a set of 10 fractions (total ~0.5 mg protein) and was removed post-digestion via acidification with trifluoroacetic acid (TFA) to a pH of ~2. The same acidification protocol was also used to precipitate the 1% SDC sample set, followed by centrifugation for sample collection. For the organic solvent analysis, a sample set was solubilized with the addition of 60% methanol. The fourth and final sample set of 10 fractions was further split into identical pairs (total ~0.25 mg protein each set) to compare vortex mixing to the administration of sonication. Both sets of these 10 fractions were solubilized with 0.15% w/v RapiGest SF in accordance with the default protocol. While one set of 10 fractions was vortexed between steps of the default digestion protocol,[49] the other set was sonicated. High-frequency sonication was performed before and between the steps of the digestion protocol using an Elmasonic P (Elma Hans Schmidbauer GmbH & Co. KG) sonicator in sweep mode at 80 kHz.

Digestion

To reduce sample variability, we injected 2 mg of protein (without enrichment) onto the first-dimension protein separation column. The resulting fractions were lyophilized and

recombined into the 10 equal mass fractions. These 10 stock fractions were split into 5 equiv sample sets of 10 fractions (each ~0.4 mg total protein) and solubilized with 1% SDC. For the digestion process, three enzymes were investigated: trypsin (TPCK-treated from Affymetrix no. 22725), chymotrypsin (TLCK treated Sigma-Aldrich no. C3142), and pepsin (Sigma-Aldrich no. P7000). While trypsin and chymotrypsin easily followed the standard in-solution digestion protocol,[49] which takes place at pH 8, solutions had to be acidified with TFA to a pH of 2 prior to the addition of pepsin. Typically this required only 1 to 2 μL of 8% TFA solution after the iodoacetamide incubation step. The trypsin and chymotrypsin were added at a ratio of 1:30 enzyme/protein, and pepsin was added at a ratio of 1:10. All incubations of single enzyme digestions took place at 37 °C overnight. A trypsin digestion at elevated temperature (60 °C) was also performed on a sample set for comparison.

In addition to the single enzyme digestions, a tandem digestion using multiple enzymes was performed. A sample set was processed using untreated trypsin (Sigma-Aldrich no. T8658), halted with acidification after overnight digestion at 37 °C, and the pH was readjusted to a pH of 7.8 using 1 M NaOH, followed by the addition of chymotrypsin for a second overnight incubation. For all digestions the reduction, alkylation, and incubation steps not described in detail were followed according to the default digestion protocol. The data processing for the tandem digestions involved selecting both a primary and secondary digest enzyme (Figure S-3) in the PLGS data-processing parameters.

Peptide Separation and Protein Identification

For the second dimension peptide separation following all digestions, the resulting peptides were analyzed and identified using a modified UHPLC coupled to MS.[52] In this modified high-pressure separation system, the LC gradient and sample are placed into a gradient storage loop using a commercial nanoAcquity UPLC (Waters). The ultra-high-pressure (30 000 psi) is achieved by pushing the gradient and sample from the loop using a pneumatic amplifier (DSHF-300, Haskel International, Burbank, CA). The peptides were separated on a 75 μm I.D. × 111 cm (manufactured in-house) capillary column with 1.7 μm 130 Å BEH C_{18} particles (Waters). The column was held at 65 °C, and the eluting peptides were detected using a QToF Premier mass spectrometer (Waters). Mobile phases consisted of water and acetonitrile with 0.1% formic acid (optima grade, Fisher Scientific), and a gradient of 4–40% organic was run over 110 min. The column was connected to a silica nanospray emitter with a 20 μm I.D. and a 10 μm tip (New Objective, Woburn, MA). The mass spectrometer was operated in MS^E mode[53] performing parent ion scans from 50 to 1990 m/z over 0.6 s at 5.0 V. The collision energy then ramped from 15 to 40 V over 0.6 s. Data were collected using MassLynx V4.1 SCN 872 and processed via ProteinLynx Global Server 2.5 (PLGS, Waters) set to a 4% false-positive rate for peptide identification with a reversed yeast proteome database obtained from UniProt protein knowledgebase (www.uniprot.org). The UniProt knowledgebase contained 7256 entries for *Saccharomyces cerevisiae* collected on 2/3/11 and was used as the basis for classification of each protein as "membrane" or "non-membrane". Proteins were classified as "membrane" if they were associated with the lipid bilayer in any way.[1] (This includes intrinsic and peripheral membrane proteins.) Proteins were classified as "non-membrane" if located in the cytoplasm, nucleus, mitochondria, and ribosomes. If the same proteins were identified in multiple fractions within a sample set, the

duplicates containing the lower identification score were not counted. For further processing parameters and a list of identified proteins, see Figure S-3. All MS data can be found at http://jjorg.web.unc.edu/following the instructions under the Shared Data tab. Once in the server, all data can be found under the Membrane Protein public file. Data are also available on www.proteomeXchange.org under the partial upload directory smMoore_PX.

RESULTS AND DISCUSSION

Cell Lysis

The lysis process was initially monitored using a microscope (400×), as it was a fast and simple way to determine whether a method was effective. For the sonication probe lysis and the freeze/thaw lysis, all cells observed beneath the microscope were still intact; therefore, these techniques were not further investigated. The cryopulverization, glass bead-beating, and French press pressure cell methods, however, contained few whole cells per magnified view. Once the membrane portions of these methods were collected, a sample from each method was diluted to equal protein concentration and digested. The results of the proteins identified are shown in Table 1. Out of the three methods, the French press cell pressure-assisted lysis technique was the most effective, and this method was used to lyse the remaining yeast for this study.

Enrichment

For the prefractionation onto the first dimension column, initial difficulties of clogging in-line filters and the column itself with membrane protein injections were remedied by placing the sample in ~80% formic acid.[11,47] This allowed for much larger volumes of intact membrane proteins to be injected onto the column for analysis and was performed despite the enrichment procedure analyzed. Three separate fractionations of equal protein concentration (0.5 mg) were performed where the membrane protein sample was injected without any enrichment, after performing acetone precipitation, and after chloroform/methanol partitioning. As shown in Figure 3, both the acetone precipitation and chloroform/methanol partitioning resulted in decreased protein identifications compared with using no prior enrichment step. Both the acetone and chloroform/methanol enrichment procedures required either precipitation or pelleting/drying of the membrane proteins. Because these techniques alter the protein's native environment, they could result in difficulty for the proteins to reconstitute prior to injection. It can be expected that once the lipid environment is removed and the proteins are precipitated, some of the membrane proteins may not redissolve into the buffered environment[10] despite the presence of formic acid prior to injection. To support this, we performed a Bradford assay before and after the acetone precipitation step, and the concentration of the sample decreased by approximately half after precipitation. This is important to realize because the protein concentration determines the amount of digestion enzyme added. If too much enzyme is added, peptides resulting from trypsin autolysis can add increased complexity for sample analysis.

Solubilization

Although sonication is traditionally used as a cell lysis step, it can be applied prior to digestion to aid with membrane protein solubilization in the presence of detergents.

Sonication applied prior to digestion moderately yet reproducibly increased the number of protein identifications compared with simply vortex mixing to improve solubilization (Table 1). The second solubilization study focused on two types of MS compatible detergents and the addition of 60% methanol. In accordance with the standard digestion protocol, 0.15% w/v of RapiGestSF was tested. Three concentrations (0.15, 1, and 5%) were previously tested for digestion efficiency to determine the appropriate concentration of SDC. Similar to reports in the literature,[9,31] the 1% concentration of SDC produced the most membrane protein identifications, while 5% showed a marked decrease. No matter the concentration of SDC (0.15, 1, or 5%), however, it consistently outperformed RapiGest SF in preparative studies. Overall, the 1% SDC identified 941 proteins (404 membrane proteins) and 0.15% RapiGest SF identified 625 proteins (279 membrane proteins) (Figure 4). In addition to more membrane protein identifications, SDC was extremely convenient, turning the solution visibly cloudy when acidification occurred, and was less expensive. Because SDC is a bile salt, becoming insoluble upon protonation, the precipitation could potentially cause protein loss; however, SDC can also be removed from solution via ethyl acetate phase transfer rather than precipitation.[4,30,32] During the phase transfer, 100 μL of ethyl acetate was added to the 100 μL of digested solution, acidified to pH ~2 with TFA, mixed, and centrifuged at 15 000g for 5 min.[30] Similar to published findings,[32] when phase transfer was compared with precipitation of SDC, both performed similarly with no marked difference in the number of proteins identified. Finally, both RapiGestSF and SDC outperformed 60% methanol for protein identifications (Figure 4). No matter the concentration of methanol attempted, the samples containing organic solvent for solubilization never outperformed any detergent tested in this study.

Digestion

Out of the three digestion enzymes tested individually (trypsin, pepsin, and chymotrypsin), trypsin outperformed all for protein identifications (Table 1). For membrane proteins, trypsin identified 228 proteins, chymotrypsin identified 12, and pepsin identified one. It is important to note that although the PLGS software allows for the selection of alternative enzymes, most statistical models for data acquisition are developed based on the behavior of tryptic peptides. Therefore, because software is designed for identifying tryptic peptides, it generally does a more successful job at finding them. It was extremely important to use fresh enzyme as well as freshly prepared chemicals during the digestion procedure (iodoacetamide, dithiothreitol, detergent, etc.), to achieve reproducible and optimal results. Tandem digestions were also attempted to increase proteome coverage. Multiple combinations of the three enzymes (trypsin–pepsin, chymotrypsin–pepsin, and trypsin–chymotrypsin) were tested prior to this study. The most promising candidate for a tandem digestion was trypsin–chymotrypsin because the use of pepsin as a secondary enzyme complicated the spectra to such an extent that many of the mass spectra collected would not complete data processing. For proteins identified for both trypsin alone and trypsin–chymotrypsin, trypsin alone produced more protein identifications (membrane and nonmembrane) and identified more digest peptides (7741 total digest peptides for trypsin and 1651 total digest peptides for trypsin–chymotrypsin). Therefore, trypsin was the digestion enzyme of choice. For the temperature of the trypsin digestion, trypsin at 37 °C identified 6 times more membrane proteins (228 membrane proteins) compared with trypsin

at 60 °C (30 membrane proteins). Also, 27 of the 30 membrane proteins identified in the high-temperature digestion were also identified at 37 °C, with only 3 unique membrane protein identifications at 60 °C. It has been reported that trypsin can experience thermal denaturation affecting stability.[54] Therefore, the digestion temperature should remain at 37 °C for the workflow.

Protocol Comparison

Overall, multiple techniques were tested and compared to determine the most efficient and reproducible method for the cell lysis, enrichment, solubilization, and digestion of membrane proteins for *S. cerevisiae* processed using the prefractionation workflow. It was determined that lysing yeast cells using a French press cell, separation of proteins without a prior enrichment step, solubilization with 1% SDC with the application of sonication, and digestion with trypsin at 37 °C provided the highest number of protein identifications. For a final comparison, this improved protocol implementing all of the selected sample preparation techniques was compared with the original default protocol previously utilized in the lab. To make an impartial comparison, we lysed cells with a French press cell and the insoluble portion of the yeast cell lysate was collected as previously described. No prior enrichment step was administered and a 1 mg protein sample was injected onto the first dimension column. The sample was fractionated and the fractions were recombined into a 10 fraction sample set so that each fraction contained an equal mass of protein per fraction (Figure 2). These 10 fractions were split into two equivalent sample sets (~0.5 mg total protein in each set). One set was processed with the improved protocol, and the second set was processed with the default protocol. Each digested fraction was run on the UHPLC–MS three times, with a protein identification resulting only if the protein was identified in at least two of the three runs. As shown in Figure 5, applying the improvements discussed increased the prefractionation workflow's membrane protein identifications by 26%. In addition, 68 membrane proteins were unique to the improved protocol, while only 18 membrane proteins were unique to the default protocol. For the membrane proteins that were identified using both protocols (176 proteins) the peptide intensity sum was higher for 93% (163 proteins) of the proteins identified by the improved method. As depicted in Figure 6, many of the proteins had more than twice the intensity when identified with the improved method. Therefore, not only did the improved protocol identify more unique membrane proteins (and peptides) but also the proteins that were identified by both methods were present in a higher abundance when applying the improved protocol. All identified proteins are listed in Figure S-4.

CONCLUSIONS

The membrane proteome can be considered the entirety of membrane proteins present in a cell at a specific time and condition.[47] Although there is no single solution for the analysis of membrane proteins, this paper demonstrated the applications of cell lysis, enrichment, solubilization, and digestion techniques for prefractionated samples of yeast to determine an expedient and reproducible workflow to increase the number of unique membrane protein identifications. It is well established that the user's optimal conditions may depend on the protein profile; however, for the model organism *S. cerevisiae* extraction via a French press

cell, no further enrichment step, solubilization with 1% sodium deoxycholate while applying sonication, and digestion utilizing trypsin at 37 °C enhanced the number of proteins identified when compared with the application of alternate sample preparation techniques. Cell lysis techniques such as high-frequency sonication, cryopulverization, freeze/thaw lysis, and bead-beating did not produce as many proteins as the application of the French press cell. Enrichment techniques such as chloroform/methanol partitioning and acetone precipitation seemed to remove proteins when compared with a sample that did not undergo an enrichment process. Although this nonenriched sample surely contained contaminants (lipids, etc.), it proved beneficial to let the sample remain in its initial state post lysis. Furthermore, solubilization with methanol and using trypsin-alternative enzymes for digestion did not prove advantageous. Overall, solubilization improvements such as utilizing sonication and sodium deoxycholate resulted in an increase in membrane and nonmembrane protein identifications for *S. cerevisiae*. Not only did this resulting improved protocol increase the unique membrane protein identifications but also 93% of the proteins commonly identified via the SOP and the improved method had a much higher intensity when identified using the improved protocol. Because this protocol was beneficial for yeast, which contains relatively tough cell membranes, the potential for its success with mammalian cells and tissue samples is expected.

Acknowledgments

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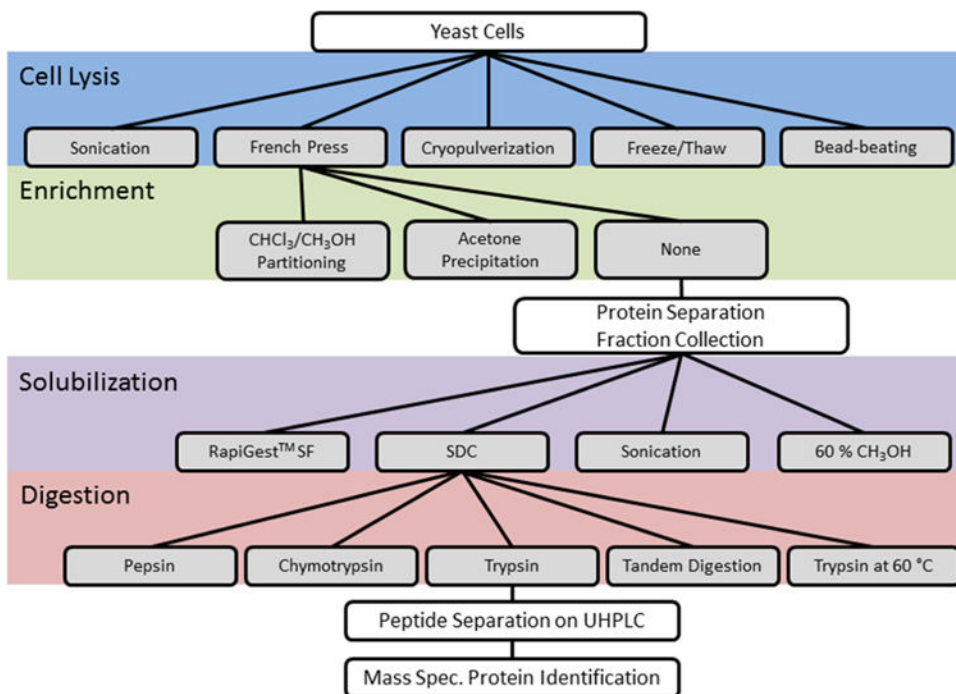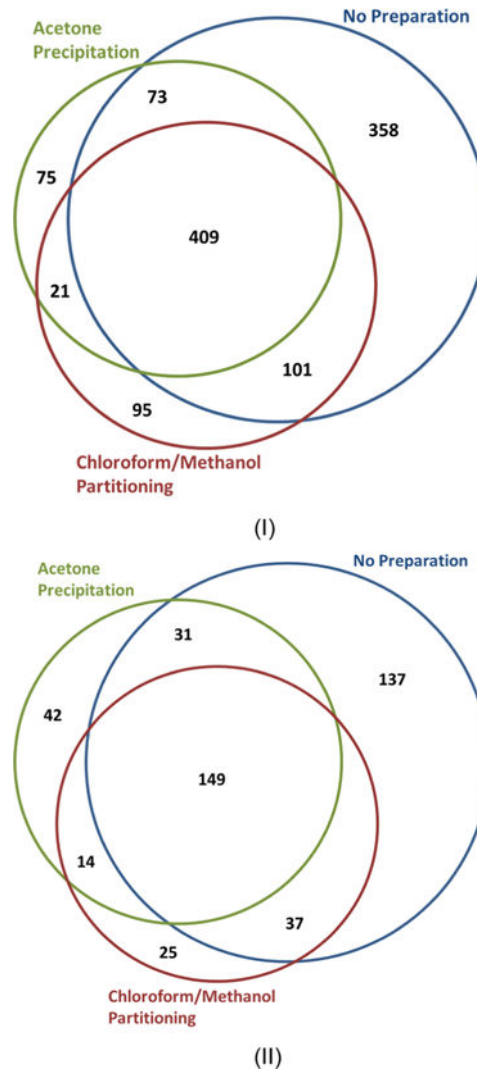
Figure 1.
Experimental outline. Sample preparation techniques from four categories (cell lysis, enrichment, solubilization, and digestion) of the membrane protein workflow were compared. Each category was tested sequentially while keeping the remaining categories constant according to the default protocol.
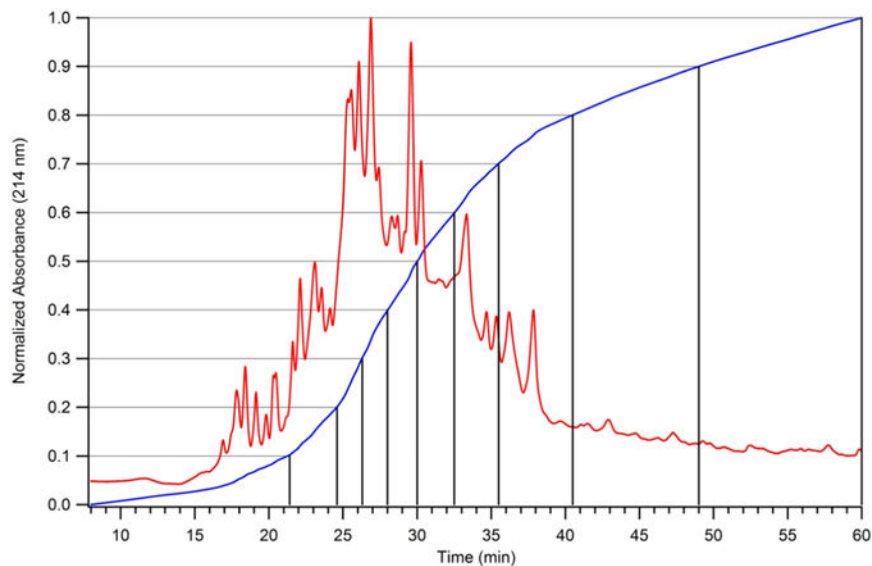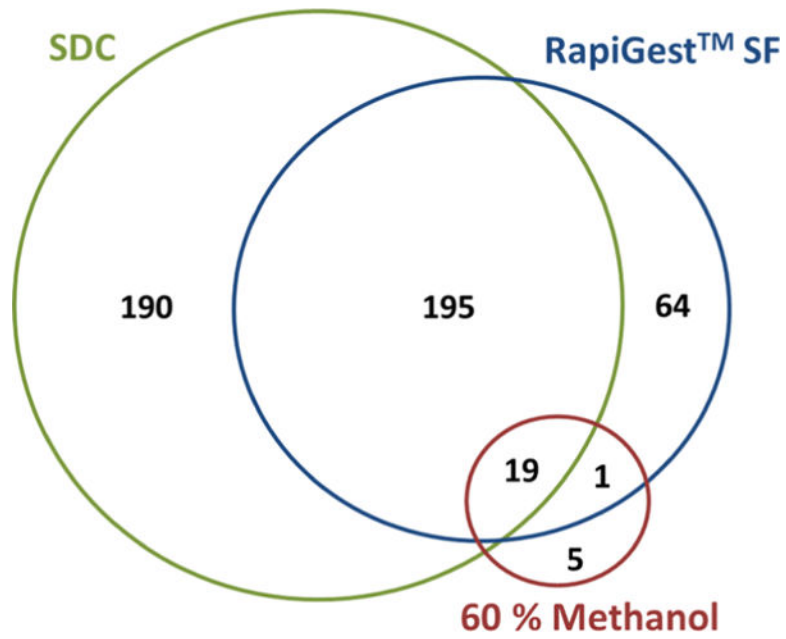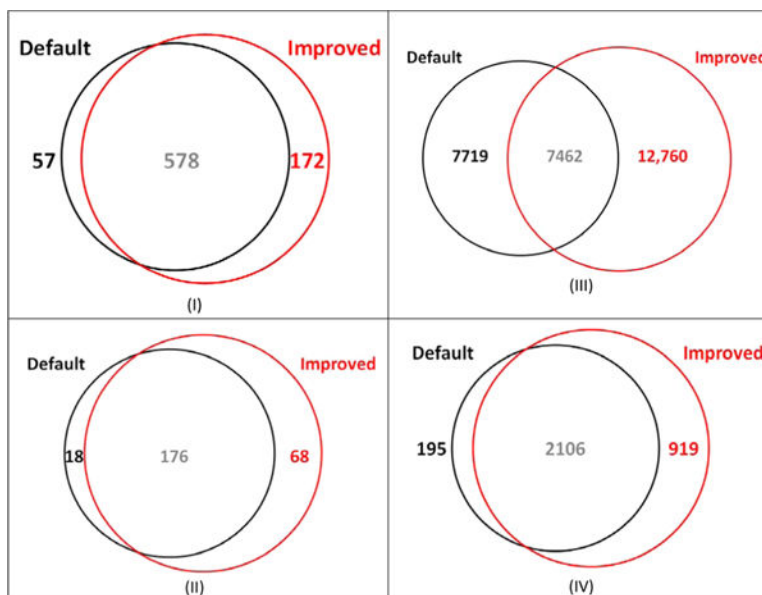
Figure 2.
Calculating equal mass fractions from equal time fractions. The UV trace for the gradient elution of intact membrane protein sample (red line), where each minute represents one fraction collected (*x* axis). The intensity of the UV trace is integrated and normalized to the most intense signal (*y* axis). The blue line represents the summed integrated area. Because this area is proportional to concentration, the area is split into the number of desired fractions along the *y* axis (e.g., 10 as shown here and used in this study). To determine which collected minute-wide fractions to combine for equal mass fraction, the *y* axis is followed over until it reaches the summed integrated area and dropped down as shown. For example, equal mass fraction 7 should contain equal time fractions 32.5–35.5. Equal time fractions are not further divided to account for this, so fraction 7 becomes 32–35 or 33–36 as per the users' discretion.
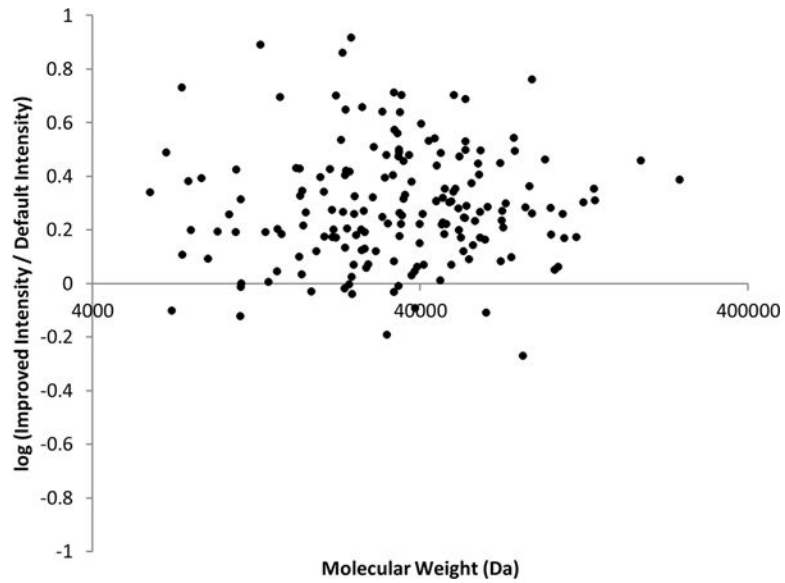
Figure 3.
Results comparing enrichment techniques: (I) total protein identifications and (II) membrane protein identifications.

Figure 4.
Membrane protein identifications comparing 1% SDC, 0.15% RapiGest SF, and 60%
methanol solubilization.

Figure 5.
Fractions collected from 0.5 mg of insoluble cell lysate were split into two sets of 10 fractions. One set was processed according to the default protocol and the other with the improved protocol. (I) Results for total protein identifications and (II) membrane protein identifications. (III) Results for total peptide identifications and (IV) membrane peptide identifications.

Figure 6.
Log of the intensity ratio versus molecular weight for the 176 membrane proteins identified using both the improved and default protocol. Proteins represented above zero (163 proteins) had a higher intensity using the improved protocol and proteins represented below zero (13 proteins) had a higher intensity using the default protocol.

Table 1

Overall Results for Extraction, Enrichment, Solubilization, and Digestion Experiments[a]

procedure	total proteins	membrane proteins	experimental details
Cell Lysis			
sonication	N/A	N/A	shotgun analysis (1D)
freeze/thaw cycles	N/A	N/A	100 μg sample
bead-beating	58	33	RapiGest SF
cryopulverization	35	17	trypsin (37 °C)
French press cell	196	80	
Enrichment			
none	941	354	fractions (2D)
acetone precipitation	578	236	0.5 mg protein
chloroform/methanol partitioning	626	225	RapiGest SF
			trypsin (37 °C)
Solubilization–Sonication Study			
vortex mixing	191	55	fractions (2D)
sonication 80 kHz sweep mode	216	60	0.25 mg protein
			no enrichment
			RapiGest SF
			trypsin (37 °C)
Solubilization–Detergents			
0.15% RapiGest SF	625	279	fractions (2D)
60% methanol	88	33	0.5 mg protein
1% sodium deoxycholate	941	404	no enrichment
			trypsin (37 °C)
			sonicated
Digestion			
high-temperature trypsin (60 °C)	101	30	fractions (2D)
pepsin	2	1	0.4 mg protein
chymotrypsin	31	12	no enrichment
trypsin	620	228	1% SDC
trypsin–chymotrypsin tandem	124	34	sonicated

[a]Experimental details held constant for each study are outlined.