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A reproducible method to enrich membrane proteins with high-purity and high-yield for an LC-MS/MS approach in quantitative membrane proteomics

Xianyin Lai*

Department of Cellular & Integrative Physiology, Indiana University School of Medicine, Indianapolis, IN 46202, USA.

Abstract

The proportionately low abundance of membrane proteins hampers their proteomic analysis, especially for a quantitative LC-MS/MS approach. To overcome this limitation, a method was developed that consists of one cell disruption step in a hypotonic reagent using liquid nitrogen, one isolation step using a low speed centrifugation, and three wash steps using high speed centrifugation. Pellets contained plasma, nuclear, and mitochondrial membranes, including their integral, peripheral, and anchored membrane proteins. The reproducibility of this method was verified by protein assay of four separate experiments with a CV of 7.7%, and by comparative LC-MS/MS label-free quantification of individual proteins between two experiments with 99% of the quantified proteins having a CV $\leq$ 30%. Western blot and LC-MS/MS results of markers for cytoplasm, nucleus, mitochondria, and their membranes indicated that the enriched membrane fraction was highly pure by the absence of, or presence of trace amounts of, non-membrane marker proteins. The average yield of membrane proteins was 237 μ g/10 million HT29-MTX cells. LC-MS/MS analysis of the membrane enriched sample resulted in the identification of 2,597 protein groups. In summary, the developed method is reproducible, produces a highly pure membrane fraction, and generates a high yield of membrane proteins.

Keywords

HT29-MTX cells; Membrane proteins; Proteomics; Reproducibility; Purity; Yield

1 Introduction

A biological membrane primarily consists of a lipid bilayer and proteins, forming an essential barrier between the cytosol and the extracellular environment, as well as compartmentalizing intracellular organelles within eukaryotes, such as nucleus, mitochondria, endoplasmic reticulum, Golgi apparatus, etc. [1]. Proteins from the plasma membrane, nuclear membrane, and mitochondrial membrane are the most frequently studied [1].

Membrane proteins can be classified into three categories according to their position relative to the lipid bilayer: integral, peripheral, and anchored. Integral membrane proteins span the

*Correspondence: Xianyin Lai, Ph.D. Department of Cellular & Integrative Physiology Biotechnology Research and Training Center Indiana University School of Medicine 1345 West 16th Street, Room 306 Indianapolis, IN 46202 USA xlai@iupui.edu Phone: 317-274-3499 Fax: 317-278-9739.

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lipid bilayer; peripheral membrane proteins are associated with the lipid bilayer surface by hydrophobic and/or ionic interactions; and anchored membrane proteins themselves are not in contact with the lipid bilayer but are attached to either side of the membrane by anchoring a covalently bound lipid group into the bilayer [2]. Integral and anchored categories can be divided into multiple subcategories according to their unique characteristics. Integral membrane proteins include type I, i.e. those that span the lipid bilayer only once with an intracellular C-terminus, type II, i.e. those that span the lipid bilayer only once with an extracellular C-terminus, and multi-pass proteins that span the membrane multiple times. Anchored membrane proteins include the lipid chain anchored proteins and the GPI (glycosylphosphatidylinositol) anchored proteins [3].

Membrane proteins are vital to various cellular processes, protecting the cell and the organelles from harmful environmental fluctuations, transporting ions, biomolecules and vesicles, transmitting electrical signals, and mediating the communication between cells and compartments [2]. A thorough knowledge of the regulation of membrane proteins is of great importance in understanding many biological processes [4]. One of the most interesting aspects of membrane proteins is the role they play in disease, transportation, signaling, and trafficking. Therefore, they are potential drug targets of interest to the pharmaceutical industry [1, 4]. As part of the cellular response to a pathological insult, membrane proteins may be secreted or shed from the cell surface into biological fluids such as plasma, cerebrospinal fluid, or urine. The measurement of membrane protein levels in these fluids can support early disease diagnosis, monitor disease severity and the progress of disease therapy, indicate the response to therapy, and act as sentinels of relapse or reoccurrence [5]. Membrane proteins represent more than one-third of the proteins encoded by the human genome; they are targets for more than two-thirds of existing drugs; and more than one-third of the current list of biomarker candidates are classified as membrane proteins [5].

Proteomic techniques, including gel-based and gel-free approaches [3-8], have been used widely in the analysis of membrane protein expression. Although it has been applied extensively to membrane proteomics, 2D gel electrophoresis suffers from substantial difficulties in resolving power and recovery [4, 9] and is not an optimal isolation technique. On the contrary, though limitations apply to shotgun proteomic analysis of membrane proteins [7], a mass spectrometry-based approach generally results in a better representation of the membrane proteome [10]. Numerous proteins have been identified from membrane fractions [11], but improvements in membrane proteomics mainly focus on increasing the number of identified membrane proteins rather than their quantification. However, because most biomarker proteins are exclusively expressed in neither the normal nor the abnormal state, comparative quantification of protein expression differences must be included in the analysis of membrane proteins [6]. Due to the fact that the membrane proteins are in low abundance proportionately to other cellular proteins, which limits their detection and identification in the presence of a high dynamic range of cellular protein expression [1], an enrichment step is essential. To obtain reliable measurements of their expression levels, an effective enrichment method should be reproducible and the enriched membrane proteins should be highly pure with high yield. In an comprehensive review by Anna E. Speers and Christine C. Wu [12], many issues related to membrane proteomics are addressed; however, reproducibility, purity, and yield were not considered. The current study presented here focused on the development of a reproducible method to enrich membrane proteins with high-purity and high-yield for an LC-MS/MS approach in quantitative membrane proteomics.

2 Materials and methods

2.1 Materials

DL-Dithiothreitol (DTT), urea, triethylphosphine, iodoethanol, and ammonium bicarbonate (NH_4HCO_3) were purchased from Sigma-Aldrich (St. Louis, MO, USA). LC-MS grade water (H_2O), LC-MS grade 0.1% formic acid in acetonitrile (ACN), and 0.1% formic acid in water (H_2O) were purchased from Burdick & Jackson (Muskegon, MI, USA). Modified sequencing grade porcine trypsin was obtained from Princeton Separations (Freehold, NJ, USA). Antibodies against β -actin (#4967), LSD1(#2184), cytochrome c (#4272), Na^+/K^+ -ATPase (#3010), COX IV (#4850), and the secondary horseradish peroxidase-conjugated goat anti-rabbit IgG antibody (#7074) were purchased from Cell Signaling Technology (Beverly, MA, USA). Antibody against Lamin B1 (ab16048) was obtained from Abcam Inc. (Cambridge, MA, USA).

2.2 Samples

HT29-MTX cells [13] (human colon adenocarcinoma cells) were kindly provided by Dr. Thécla Lesuffleur (INSERM, Paris, France) and were cultured in T75 flasks in Dulbecco's modified Eagle's medium (DMEM; Invitrogen, Carlsbad, CA, USA) supplemented with 10% heat inactivated fetal bovine serum (Hi-FBS; Invitrogen, Carlsbad, CA, USA) in a humidified incubator with 5% CO_2 at 37 °C. The cells were allowed to grow to 90% confluence, dissociated with a solution of 0.25% trypsin/EDTA, centrifuged at $120 \times g$ for 5 min, resuspended with 2 mL fresh medium, manually counted under a microscope using a hemocytometer, aliquoted 10 million cells for each sample, washed with PBS twice, and centrifuged at $200 \times g$ for 2 min.

2.3 Membrane Protein Enrichment

This procedure includes a cell disruption step and multiple wash steps (Fig. 1). After 500 μL of H_2O were added to a sample (10 million cells), the sample was pipetted up and down 10 times, incubated for 10 min on ice, frozen for 1 min in liquid N_2 , thawed at room temperature, and centrifuged for 10 min at $10,000 \times g$, 4 °C. After 500 μL of H_2O were added to the pellets, the sample was pipetted up and down 20 times, incubated for 10 min on ice, centrifuged for 20 min at $100,000 \times g$, 4 °C. After multiple wash steps, 500 μL of 8 M urea with 10 mM DTT were added to the pellets. Fully solubilized samples were then stored at -80 °C until analysis. Protein concentration was determined by the Bradford Protein Assay using Bio-Rad protein assay dye reagent concentrate [14].

Eight samples (A-H) were used in this study. Sample A was solubilized using 500 μL of 8 M urea with 10 mM DTT without membrane protein enrichment; Samples B - H were washed 0 - 6 times, respectively. Because different wash steps were applied and the labeling of the fractions is a little complicated, they are listed in Supplementary Table 1.

2.4 SDS-PAGE and Western Blotting

SDS-PAGE and Western transfer were carried out according to the manufacturer's instructions for NuPAGE® Novex Midi Bis-Tris gel. Briefly, 10 μL of each fraction were mixed with 5 μL of NuPAGE® LDS Sample Buffer (4X) (NP0007; Invitrogen), 1.5 μL of NuPAGE® Reducing Agent (10X) (NP0009; Invitrogen), and 3.5 μL of H_2O . Samples were heated at 70 °C for 10 min and run on NuPAGE® Novex 8 % and 4-12% Bis-Tris Midi Gels (WG1001BOX; WG1401BOX; Invitrogen) in NuPAGE® MOPS and MES SDS Running Buffers (NP0001; NP0002; Invitrogen) with NuPAGE® Antioxidant (NP0005; Invitrogen). The detailed SDS-PAGE gels, buffers, and other related information for each protein are presented in Supplementary Table 2. The separated proteins were transferred to Immobilon-P Transfer Membrane (IPVH00010; Millipore) at 25 V for 1 h with NuPAGE® Transfer

Buffer (NP0006-1; Invitrogen). After 2 h incubation in PBS with 0.3% Tween® 20, various organelle marker protein antibodies were added and incubated overnight at 4 °C, including β -actin, LSD1, and cytochrome c for cytoplasm, nucleus, and mitochondria, respectively; Na⁺/K⁺-ATPase, Lamin B1, and COX IV for plasma, nuclear, and mitochondrial membrane, respectively. After washing with 0.3% Tween® 20 in PBS every 15 min four times, the secondary horseradish peroxidaseconjugated goat anti-rabbit IgG antibody (1:1000; #7074; Cell Signaling Technology, Beverly, MA, USA) were added and incubated at room temperature for 2 h. The membrane was washed for 15 min using PBS with 0.3% Tween® 20 for four times, the blots were developed with Pierce® ECL Western Blotting Substrate (32209; Thermo Scientific), and reactive bands were detected by exposure to CL-XPosure Film (34090; Thermo Scientific) at ambient temperature.

2.5 Sample Preparation for Proteomic Analysis

Two experiments were carried out. In the first experiment, one sample was used for protein identification only. In the second experiment, two samples were used to evaluate the reproducibility of the enrichment method. Protein reduction, alkylation, and digestion were carried out using a conventional method previously published by the author [15]. Briefly, a 100 μ g aliquot of enriched membrane protein sample was placed in a 2 mL tube. The volume of the sample and concentration of urea were adjusted to 200 μ L and 4 M urea. 200 μ L of the reduction/alkylation cocktail consisting of 0.5% of triethylphosphine and 2% of iodoethanol was added to the protein solution. The sample was incubated at 35°C for 60 min, dried by SpeedVac, and reconstituted with 100 μ L of 100 mM NH₄HCO₃ at pH 8.0. A 150 μ L aliquot of a 20 μ g/mL trypsin solution was added to the sample and incubated at 35°C for 3 h, after which another 150 μ L of trypsin was added, and the solution incubated at 35°C for 3 h.

2.6 LC-MS/MS

Two LC-MS/MS experiments were carried out. In the first experiment, one sample was used, and 13 injections of the sample were analyzed for protein identification only. In the second experiment, two samples were used, 7 injections of each sample were analyzed, and the comparison of two samples was carried out to evaluate the reproducibility of the enrichment method. The digested samples were analyzed using a Thermo-Finnigan linear ion-trap (LTQ) mass spectrometer coupled with a Surveyor autosampler and MS HPLC system (Thermo-Finnigan). Tryptic peptides were injected onto a C18 reversed phase column (TSKgel ODS-100V, 3 μ m, 1.0 mm $\times$ 150 mm) at a flow rate of 50 μ L/min. The mobile phases A, B, and C were 0.1% formic acid in water, 50% ACN with 0.1% formic acid in water, and 80% ACN with 0.1% formic acid in water, respectively. The gradient elution profile was as follows: 10% B (90% A) for 7 min, 10-67.1% B (90-32.9% A) for 163 min, 67.1-100% B (32.9-0% A) for 10 min, and 100-50% B (0-50% C) for 10 min. The data were collected in the “Data dependent MS/MS” mode with the ESI interface using normalized collision energy of 35%. Dynamic exclusion settings were set to repeat count 1, repeat duration 30 s, exclusion duration 120 s, and exclusion mass width 0.60 m/z (low) and 1.60 m/z (high).

2.7 Protein Identification and Quantification

The acquired data were searched against the International Protein Index (IPI) human database (ipi.HUMAN.v3.83) using SEQUEST (v. 28 rev. 12) algorithms in Bioworks (v. 3.3). General parameters were set to: mass type set as “monoisotopic precursor and fragments”, enzyme set as “trypsin(KR)”, enzyme limits set as “fully enzymatic - cleaves at both ends”, missed cleavage sites set at 2, peptide tolerance 2.0 amu, fragment ion tolerance 1.0 amu, fixed modification set as +44 Da on Cysteine, and no variable modifications used. The cut-off of SEQUEST scores, such as XCorr (Cross-correlation) and Δ Cn (the difference

between the first and second-ranked sequences) was not employed. The searched peptides and proteins were validated by PeptideProphet [16] and ProteinProphet [17] in the Trans-Proteomic Pipeline (TPP, v. 3.3.0) (<http://tools.proteomecenter.org/software.php>). Only proteins and peptides with protein probability 0.9000 and peptide probability 0.8000 were reported. FDR was not applied and the reasons are described in Supplementary Discussion 1. After TPP validation, proteins identified by one peptide were included. Protein quantification was performed using a label-free quantification software package, IdentiQuantXL™ [18].

2.8 Bioinformatics

To ascertain the percentage of membrane proteins in the enriched samples, an examination of all detected proteins was carried out using Generic Gene Ontology (GO) Term Mapper (<http://go.princeton.edu/cgi-bin/GOTermMapper>) with settings of Goa_human (GOA GO slim) as GO Slim and GO:0016020 (membrane) as GOID.

3. Results and discussion

3.1 Cell Disruption

Cell disruption is the initial step in membrane enrichment. Various methods have been reported [19, 20], but specific methods must be chosen based on the explicit aim of each experiment and the downstream applications that follow cell disruption. The aim of this study was to develop a method to enrich total membrane proteins, including the plasma, nuclear, and mitochondrial membrane proteins for quantitative LC-MS/MS analysis. Accordingly, reproducibility was one of the most important concerns for choosing the various components of the overall approach.

Chemically-based cell disruption methods are not optimal choices due to the following reasons. Alkali treatment, organic solutions, and detergents dissolve the membrane, causing difficulties in the isolation of membrane protein from cytoplasmic proteins. Enzymatic digestion and saponin treatment are limited to certain types of cells.

Considering the reproducibility issue, many physical methods are not optimal choices either. Bead beating, Polytron® homogenizer, Dounce® homogenizer, needle, nitrogen cavitation, and French press all create reproducibility issues due to an inability for batch processing. On the contrary, sonication is amenable to batch processing, but it produces moderate to fine cell debris, causing irreproducible membrane isolation in downstream steps.

An effective cell disruption method should capably process samples in a batch and break membranes into large fragments. Osmotic shock is one of these methods, using a hypotonic medium to disrupt the plasma membrane by osmotic pressure. However, it is very gentle and generally restricted to the disruption of red blood cells [21, 22]. Initially, HT29-MTX cells were tested using a hypotonic medium at the beginning of method optimization. After 500 µL of water were added to 10 million HT29-MTX cells, the sample was pipetted up and down 10 times, and incubated for 10 min on ice. Microscopic examination indicated that many cells remained intact, suggesting that osmotic shock alone is not strong enough to suitably disrupt the HT29-MTX cell membranes. It is likely that this method alone may be insufficient for other cell lines, as well.

Freeze-thaw is an alternative disruption method. According to the literature and our own pilot results, freeze-thaw is more effective in cell disruption when combined with exposure to a hypotonic solution, e.g. water. For example, when liquid N₂ was used to rupture *E. coli* suspended in 10 ml of ice-cold 50 mM Tris-HCl (pH 8.0) supplemented with 100 mM DTT and Complete protease inhibitor mixture tablets, unbroken cells remained [23],

substantiating the inefficiency of liquid N₂ alone to achieve complete disruption. Furthermore, the temperature and speed of freezing seem to be key factors that influence its effectiveness. In a study where cells were suspended in hypotonic buffer and processed by five cycles of freezing in -80 °C and thawing in a RT water bath, microscopy revealed that about 10% of the cells were not disrupted [24]. In another study, a dry ice/ethanol bath was used to freeze red blood cells in hypotonic lysis buffer [25]. We tested this method using HT29-MTX cells. Western blot results revealed that LSD1, a nuclear marker protein, was highly abundant in the membrane fraction, indicating the dry ice/ethanol bath is not effective in disrupting the nucleus (Supplementary Fig. 1). Protein assay results indicated that the percentage of membrane proteins generated using the dry ice/ethanol bath was much higher than the percentage using liquid N₂ (23.4% vs. 13.9%) (Fig. 2). This is consistent with the Western blot result and likely due to unbroken cells that contaminated the membrane fraction.

In our study, pure water, without any additional reagents, was used as the hypotonic agent, thereby combining osmotic shock and liquid N₂ freeze-thaw. No such cell disruption method has been reported previously, though a similar method has been published [26]. In that study, cells were disrupted by freeze-thaw in hypotonic buffer using liquid N₂, with a subsequent step of detergent disruption. However, no additional membrane isolation and purification steps as we describe below were included in their approach.

3.2 Membrane Isolation

Following cell disruption, membrane isolation is the second step in membrane protein enrichment. Two-phase partitioning has been used to separate membrane from cytoplasmic proteins [27]. However, this approach requires subsequent phase isolation and transference, making reproducibility difficult to achieve. Centrifugation is the predominant approach applied in membrane isolation, yielding reproducible membrane protein samples. Thus, centrifugation was chosen in our approach.

When membrane protein yield is considered, one aspect of the centrifugation approach, i.e. fraction collection, becomes problematic. Many previous investigators have ignored this key issue. For instance, in one such investigation, cells were disrupted by freeze-thaw at -80 °C. After removal of non-lysed cells from total cell lysates by 1,000 ×g centrifugation (6 min, 4°C), samples were subjected to 100,000 ×g centrifugation (1 h, 4°C) and the pellets containing membrane proteins were collected [24]. In another study, the cells were ruptured by liquid N₂ cracking, the unbroken cells were removed by centrifugation at 5,000 ×g, and the supernatant was collected for further membrane protein enrichment [23]. In both cases, the supernatant fraction was collected and the pellet discarded in the first centrifugation step. Unfortunately, large membrane fragments were pelleted and removed along with the whole cells, resulting in a low yield of membrane proteins in the supernatant.

With respect to purity, centrifugation speed is another important factor in membrane protein isolation. In the present study, a low speed centrifugation (10K ×g) was applied in the first step to collect all the fragments of plasma, nuclear, and mitochondrial membranes. Because all cells, nuclei, and mitochondria were disrupted into large fragments, a low speed should be able to pellet them without contamination by other organelles. To test the effect of different centrifugation speed on membrane isolation, 0.6, 1.0, 2.0, 3.0, 5.0, and 10 K ×g were evaluated. The membrane marker Western blot results indicated that the 0.6 K spin speed was able to pull down the membranes. However, when lower speeds (< 5K ×g) were applied, plasma and mitochondrial membrane proteins were detectable in the supernatants (Supplementary Fig. 2), indicating incomplete membrane pelleting, a source of significant variability. Due to its ability to pull down all the membranes and its practicality in most biomedical laboratory centrifuges, 10 K ×g was chosen.

In the three subsequent wash steps, a high speed centrifugation ($100K \times g$) was used to isolate membrane proteins from soluble proteins. Because large membrane fragments were observed to become fine debris during the wash steps causing the solution in the lower part of the centrifuge tube to become cloudy, a low speed spin was deemed insufficient to pellet them. Accordingly, a high speed must be applied in the wash steps.

3.3 Membrane Purification

After the membranes are isolated, the removal of proteins nonspecifically bound to them is required. To determine the number of washes required to achieve optimal purity, Western blotting was applied to evaluate the results of each method. In the comparison, 8 samples were used, one sample per method. Sample A was a whole cell lysate without membrane isolation; sample B included only one membrane isolation step; samples C-H included one membrane isolation step and 1-6 wash steps, respectively. The samples generated in all steps are listed in Supplementary Table 1. The Western blot results are shown in Figure 3. β -actin, LSD1, and cytochrome c are markers for cytoplasm, nucleus, and mitochondria, respectively. Na^+/K^+ -ATPase, Lamin B1, and COX IV are markers for plasma, nuclear, and mitochondrial membrane, respectively. Without any wash steps (Samples B and B1), β -actin, LSD1, and cytochrome c were abundant in the membrane protein fractions, indicating that non-membrane proteins were significant contaminants. With incremental wash steps, their abundance decreased. After three wash steps, the presence of β -actin and LSD1 in the membrane fraction (Sample E) was very low, and cytochrome c was not detectable (Sample E) by Western blot. Therefore, three wash steps were chosen for our membrane enrichment method.

3.4 Reproducibility

Sample reproducibility is one of the most important concerns for effective quantitative LC-MS/MS analysis. To investigate the reproducibility of the enrichment method, four HT29-MTX cell samples, 10 million of cells in each sample, were collected and their membrane proteins enriched on four different days. Protein assay results for each fraction and their percentages are shown in Figure 4. Total protein in the four samples was 1687.1, 1780.1, 1583.9, and 1939.1 μ g. These amounts were highly consistent, and similar to the percentage of membrane protein fractions, e.g. 13.3, 14.4, 12.0, and 13.9%. The CVs of the total protein and membrane protein yields were 8.6 and 7.7 %, respectively, indicating this membrane enrichment method is highly reproducible. Although the percentages of membrane fractions depicted in Figure 4 appear to be highly reproducible, the other fractions are substantially less reproducible than membrane results. The reasons are described in Supplementary Discussion 2.

To evaluate the reproducible enrichment of individual membrane proteins, a comparison of quantitative LC-MS/MS analysis of two samples generated on 06/14/2011 and 07/05/2011 (see the labels in Fig. 4) was carried out. From the 7 injections of each sample (14 injections in total), 2,362 unique protein groups were quantified. The intensity of each protein in each injection, the average intensity of each protein in each sample, and the CV of each protein's average intensity between the two samples are listed in the Supplementary Table 3. According to Food and Drug Administration (FDA) Guidance documents, the acceptance criteria of assay performance for small molecule assays are set at a CV $\pm$ 15% as the default value ($\pm$ 20% at the lower limit of quantitation, LLOQ) [28]. The suggested acceptance criteria of assay performance for biomarker assays are set at a CV $\pm$ 25% acting as default value ($\pm$ 30% at the LLOQ) [29]. Thus, 15, 20, 25, and 30% were applied to sort the CVs of the proteins. Among the 2,362 proteins, 2,001 proteins (85%) were quantified with a CV 15%, 200 proteins (8%) were quantified with a CV > 15% and 20%, 91 proteins (4%) with a CV > 20% and 35%, 41 proteins (2%) with a CV > 25% and 30%, and only 29

proteins (1%) with a CV > 30% (Fig. 5A). The individual protein CV analysis clearly shows 99% of the proteins were quantified with an acceptable CV, verifying the reproducibility of this enrichment method. The intensity and CV of the six marker proteins are presented in Figure 5B. Membrane protein markers Na⁺/K⁺-ATPase (ATP1A1), Lamin B1 (LMNB1), and COX IV (COX4I1) were quantified with a CV of 6, 7, and 13%, respectively. Non-membrane protein markers β -actin (ACTB) and LSD1 were not detected in the membrane protein enrichment sample. Mitochondrial marker protein cytochrome c (CYCS) was detected and quantified with a CV of 4%. MS quantification data further verified the reproducibility by calculation of each protein's CV.

3.5 Purity and Yield

The purity of the enriched membrane proteins was investigated using Western blotting. As shown by sample E in Figure 3, each fraction is highly pure, except that trace amounts of β -actin and LSD1 are detected in the membrane fractions due to their high abundance. Additionally, immunodetection is very a sensitive technique, enabling the detection of very low protein quantities. These results indicated that only trace amounts of cytosolic proteins existed in the membrane protein fraction and verified that this fraction is highly pure.

LC-MS/MS analysis of the enriched samples provides another angle from which to view purity. As shown Figure 5B, cytoplasm marker protein β -actin (ACTB) and nucleus marker protein LSD1 were not detected in the membrane protein enrichment sample. Although mitochondrial marker protein cytochrome c (CYCS) was detected, its intensity is only 15% of the mitochondrial membrane protein marker COX IV (COX4I1). In general, it is difficult to determine whether Western blot or LC-MS/MS is more sensitive. Nonetheless, the consistent results generated from the enriched samples by both techniques indicate that the membrane fraction is highly pure.

According to the results of four unique experiments performed on different days, the average yield of membrane proteins was 237 μ g/10 million cells. The yield is much higher than a reported value [30], 30 - 130 μ g/10 million cells, achieved by a commercial kit that presented the highest purity among the five tested kits.

Many membrane protein enrichment methods produce highly pure samples but with low yield, other methods generate high yield of membrane proteins but with low purity. The method described here enriches membrane proteins with high purity and high yield. The key aspect to this novel approach is the disruption of all membranes into large fragments instead of fine debris and the collection of these large fragments using a low speed centrifugation.

3.6 Bioinformatics

In the identification experiment, because proteins identified from the same peptides are counted as one group, 2,597 unique protein groups (5,717 proteins) were identified from the 13 LC-MS/MS injections of the enriched sample (Supplementary Table 4). Using GO Term Mapper with settings of Goa_human (GOA GO slim) as GO Slim and GO:0016020 (membrane) as GOID, 123 out of the 5,717 were found to be ambiguous, 2,065 were found to be unannotated, 1,780 were not annotated in GO slim but had non-root annotations that were not in the slim, 4 had no non-root annotations, and 1,745 were annotated as 'membrane' in the GO ontology, indicating that 49.45% (1745 of 3529) of those with a GO compartment annotation of 'membrane'.

The ratio of membrane protein to total protein is not extremely high because the membrane protein sample includes peripheral membrane proteins. Many proteins (39%) are located in multiple organelles and imported into the membrane, the identification of a protein "traditionally" localizing in plasma, does not automatically exclude its membrane

localization [5], especially for peripheral membrane proteins. The GO ontology database does not contain all compartment annotations of a protein, especially for peripheral membrane proteins. This may explain why the ratio of membrane protein to total proteins in our project is slightly lower than that from the alkaline carbonate processed samples.

4 Concluding remarks

Reproducibility, purity, and yield are the most important considerations in the development of membrane protein enrichment methodology. These issues determine the strategies and methods applied in cell disruption, membrane isolation, and membrane purification. The approach presented here is specifically directed at the comparative quantification of membrane protein using LC-MS/MS analysis, a strategy requiring high reproducibility in sample preparation. In addition, other factors affect one's choice of methods including simplicity, processing time, costs, recovery volume, and reagents impacting the workflow for downstream analyses. We have demonstrated a new method that 1) is reproducible, 2) produces a highly pure membrane fraction, 3) generates a high yield of membrane proteins, 4) is very simple, 5) requires a short processing time, and 6) is low cost.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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Abbreviations

GPI	glycosylphosphatidylinositol
GO	Gene Ontology

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Fig. 1.

An example of the typical workflow for sample H that was washed 6 times. This procedure includes a cell disruption step and six wash steps, generating samples H1-7 and membrane fraction sample H. After 500 μL of H_2O were added to 10 million cells, the sample was pipetted up and down 10 times, incubated for 10 min on ice, frozen for 1 min using liquid N_2 , thawed at room temperature, and centrifuged for 10 min at $10,000 \times g$, 4°C . The supernatant was removed. In wash steps, after 500 μL of H_2O were added to the pellets, the sample was pipetted up and down 20 times, incubated for 10 min on ice, centrifuged for 20 min at $100,000 \times g$, 4°C . After multiple wash steps, 500 μL of 8 M urea with 10 mM DTT were added to the final pellets.

Fig. 2.

Evaluation of the efficiency of freeze-thaw using a dry ice/ethanol bath versus liquid N₂. The comparison reveals that the ratio of membrane protein to total protein generated using the dry ice/ethanol bath is much higher than that obtained using liquid N₂ (23.4% vs. 13.9%). This is consistent with the Western blot result because unbroken cells contaminated the membrane fraction. E1, Supernatant from the cell disruption step; E2, Supernatant from the first wash step; E3, Supernatant from the second wash step; E4, Supernatant from the third wash step; E, Pellets of membrane protein fraction.

Fig. 3. Comparison of multiple wash steps using Western blotting. In the comparison, 8 samples were analyzed, generating 36 subsamples, as listed in Supplementary Table 1. Sample B includes only one membrane isolation step; samples C-G include one membrane isolation step and 1-5 wash steps, respectively. β -actin, LSD1, and cytochrome c are markers for cytoplasm, nuclear, and mitochondria, respectively. Na^+/K^+ -ATPase, Lamin B1, and COX IV are markers for plasma, nuclear, and mitochondrial membrane, respectively. Without wash steps (Samples B and B1), β -actin, LSD1, and cytochrome c were abundant in the membrane protein fractions, indicating that non-membrane proteins contaminated the membrane fraction. With additional wash steps, their abundance decreases. After three wash steps, the abundance of β -actin and LSD1 in the membrane fraction (Sample E) is very low, and cytochrome c is undetectable in the membrane fraction (Sample E). Therefore, three wash steps were chosen for the membrane enrichment method.

Fig. 4. Reproducibility of the approach. Carried out on different days, the total protein measurements of 10 million HT29-MTX cells are 1687.1, 1780.1, 1583.9, and 1939.1 μg , and the membrane protein fractions are 13.3, 14.4, 12.0, and 13.9%. The CV of the total protein and membrane protein yields are 8.6 and 7.7 %, respectively, indicating the membrane enrichment method is highly reproducible. E1, Supernatant from the cell disruption step; E2, Supernatant from the first wash step; E3, Supernatant from the second wash step; E4, Supernatant from the third wash step; E, Pellets of membrane protein fraction.

Fig. 5.

Quantitative LC-MS/MS analysis. (A) Among the 2,362 proteins, 2,001 proteins (85%) were quantified with a CV ≤15%, 200 proteins (8%) were quantified with a CV >15% and ≤20%, 91 proteins (4%) with a CV >20% and ≤25%, 41 proteins (2%) with a CV >25% and ≤30%, and only 29 proteins (1%) with a CV >30%. The individual protein CV analysis clearly shows 99% of the proteins were quantified with an acceptable CV, verifying the reproducibility of the enrichment method. (B) The intensity and CV of the six marker proteins are presented. Membrane protein markers Na⁺/K⁺-ATPase (ATP1A1), Lamin B1 (LMNB1), and COX IV (COX4I1) were quantified with a CV of 6, 7, and 13%, respectively. Non-membrane protein markers β-actin (ACTB) and LSD1 were not detected in the membrane protein enrichment sample. Mitochondrial marker protein cytochrome c (CYCS) was detected and quantified with a CV of 4%, but its intensity is only 15% of the mitochondrial membrane protein marker COX IV (COX4I1). The data in Figure 5B also are consistent with the purity analysis data in Figure 3, indicating that the membrane fraction is highly pure.